Molecular characterisation of surface antigens of Enterococcus faecalis in infective endocarditis

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Summary.

Enterococcus faecalis causes 5-15% of cases of infective endocarditis, and is a particularly difficult organism to treat. In previous studies, three surface antigens (37, 40 and 73 kDa) have been identified, which appear specific for *E. faecalis* infective endocarditis (EFIE). Antibodies against these antigens were detected only in serum from patients with EFIE and not in serum from patients with endocarditis due to other organisms, or *E. faecalis* infections at other sites.

A λZAPII library, constructed from *E. faecalis* EBH1 genomic DNA, was screened using serum from an EFIE patient with high titres of antibodies against the 37, 40 and 73 kDa antigens. Antibody affinity purification using one strongly reacting plaque revealed that DNA encoding a protein cross reactive with both the 37 and 40 kDa antigens had been cloned. Sequence analysis of the insert showed the presence of an open reading frame comprising 308 codons encoding a polypeptide of molecular weight 34768 Da which was designated *Enterococcus faecalis* antigen A (EfaA). Amino acid sequence alignment revealed 55-60% homology with the adhesins FimA, SsaB, ScaA and PsaA from *S. parasanguis, S. sanguis, S. gordonii* and *S. pneumoniae*, respectively. A 0.4 kb HindIII fragment of *efaA* hybridised to all *E. faecalis* strains tested, but failed to hybridise to genomic DNA from other streptococci. Northern analysis revealed that *efaA* was transcribed on an approx 3 kb message, and that expression was regulated by some component(s) of serum.

For Mum and Dad.

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Abbreviations.

AMPS	ammonium persulphate
ATP	adenosine triphosphate
BATH	bacterial adherence to hydrocarbon
BCIP	5-bromo-4-chloro-3-indoyl phosphate (X-phosphate)
BSA	bovine serum albumin
cm	centimetre(s)
Da	daltons
DEPC	Diethyl pyrocarbonate
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EDTA	ethylene diamine tetra-acetic acid
ESR	erythrocyte sedimentation rate
FCS	foetal calf serum
g	acceleration due to gravity (= $9.81 ms^{-2}$)
g	gramme(s)
IPTG	isopropyl β-D-thiogalactopyranoside
l	litre(s)
LB	Luria broth
M	mole(s) per litre
MIC	minimum inhibitory concentration
μ	micro
mg	milligram(s)
ml	millilitre(s)
m.o.i.	multiplicity of infection
NBT	nitro blue tetrazolium
nm	nanometre(s)
OD	optical density
ORF	open reading frame
pfu	plaque forming units
PIPT	pheromone-inducible plasmid transfer
PMN	polymorphonuclear leukocyte
RNA	ribonucleic acid
rpm	revolutions per minute
Sarkosyl	N-lauroylsarcosine, sodium salt
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
TEMED	N,N,N,'N'-Tetramethylethylene diamine.
Tris	tris (hydroxymethyl) aminomethane
UTI	urinary tract infection
V	volt(s)
v/v	volume by volume
w/v	weight by volume
X-gal	5-Bromo-4-Chloro-3-Indoyl-β-D-Galactopyranoside
YE	yeast extract

Chapter 1: Introduction.

1.1. Infective endocarditis.

Infective endocarditis is the result of microbial colonisation of the endocardium. The valves are most commonly affected, although the disease can also affect the mural endocardium or septal defects. Healthy heart tissue is not normally susceptible to infection, however damage to the endocardium initiates a chain of events leading to the formation of a non-bacterial thrombotic endocarditis (NBTE) which pre-disposes to colonisation. Patients with prosthetic heart valves are also at risk. The disease has been classified according to its progression as acute (death in less than 6 weeks), subacute (death in 6 weeks to three months) and chronic (death in greater than three months). However, these terms have more recently given way to classification according to aetiologic agent, which has more bearing on the appropriate treatment (Scheld and Sande, 1990).

1.1.1. Aetiology.

Infective endocarditis can be caused by a range of micro-organisms including mycobacteria, fungi, chlamydiae, rickettsiae, viruses and Gram-negative bacteria. However, aetiologic diagnosis of infective endocarditis, as determined by blood cultures reveals that the vast majority of cases arise due to infection by Gram-positive cocci. Figures vary according to the surveys used, but streptococci (mainly *S. sanguis*, *S. bovis*, *S. mutans* and *S. mitior*) usually account for at least half of all cases, staphylococci for 20-30% (predominantly coagulase positive *S. aureus*), and enterococci (mainly *E. faecalis*) for 5-18% of cases (Scheld and Sande, 1990; Barco, 1991; Watanakunakorn and Burkert, 1993). Gram-negative bacteria, which are rarely isolated (<5% of cases), include *E. coli*, *Salmonella* sp. and the HACEK group

(*Haemophilus, Actinobacillus, Cardiobacterium, Eikenella and Kingella*; Scheld and Sande, 1990).

1.1.2. Incidence.

Most surveys of infective endocarditis report that its incidence has not changed for 30 to 40 years. It is difficult to assign a definite figure for the incidence of the condition since diagnostic criteria vary. Indeed, applying strict criteria has revealed that as few as 20% of clinically diagnosed cases can be categorised as definite (Scheld and Sande, 1990). Incidences quoted recently include, in England and Wales, 1000-2000 cases per year or 20 cases per million of the population (Littler and Shanson, 1989), 10,000-15,000 new cases per year in the USA (Bayer, 1993), or 4.9 cases per 100,000 person-years (Steckelberg *et al*, 1990). Endocarditis is most likely to affect the left side of the heart, the mitral valve being affected more often than the aortic valve. Combined aortic and mitral valve infection is less common (Watanakunakorn and Burkert, 1993).

1.1.3. Mortality.

The mortality of endocarditis depends upon the site of involvement, the aetiologic organism, host risk factors and the number of complications (Lukes and Durack, 1993). A ten year study of infective endocarditis by Watanakunakorn and Burkert (1993) showed an overall mortality of 21.4%, with most deaths being due to *S. aureus*. Mortality was significantly higher among the elderly (10.1% under 60 years, 31.5% over 60 years), and was lower among patients with prosthetic valve endocarditis (PVE) than with native valve endocarditis (NVE). The lower mortality among cases of PVE has also been noted in studies of enterococcal endocarditis (Almirante *et al*, 1991; Rice *et al*, 1991), and has been attributed to the shorter period of disease before diagnosis of PVE. Aortic valve involvement is regularly associated with unfavourable prognosis, and the need for surgery (Rice *et al*, 1991; Almirante *et al*, 1991; Lukes and Durack, 1993). The effect of antimicrobial therapy on the

outcome of enterococcal endocarditis in severall studies was examined by Megran (1992), who found that with standard therapy (β-llactam plus aminoglycoside) average mortality was 17% (0-43%). However, in cases where non-standard or inappropriate therapy was used, 46% (17-100%) of patients died. These findings highlight the effect that antimicrobial therapy has had upon the mortality of infective endocarditis, and underline the need to select the appropriate treatment.

The work described in this thesis involves *Enterococcus faecalis* endocarditis therefore the literature relating to endocarditis caused by *E. faecalis* and associated organisms will be discussed below.

1.2 The streptococci.

Streptococci are facultatively anaerobic, Gram-positive, spherical or ovoid bacteria (0.5-2.0 μm in diameter), which grow as pairs or chains in liquid media. Streptococci commonly attack erythrocytes (haemolysis), and may be characterised as α-, β- or γ-haemolytic, according to their appearance on blood agar. Alpha-haemolytic streptococci produce partial haemolysis (viridans streptococci), beta-haemolytic streptococci produce a clear zone of complete haemolysis, whilst gamma-streptococci are non haemolytic (Holt *et al*, 1994). Following her discovery of group specific antigens, Lancefield (1933) used serological typing to divide the genus into approximately 20 groups, each of which were assigned a letter of the alphabet. This system is no longer considered altogether reliable, with many serological groups overlapping (Gallis, 1990; Holt *et al*, 1994).

1.2.1. Group D streptococci.

Group D streptococci are mainly non-haemolytic, and are distinct from other streptococcal groups in their ability to grow in medium containing 40% bile, and to cleave esculin. Traditionally, group D streptococci were separated into enterococcal

(eg *S. faecalis*, *S. faecium* and *S. durans*), and non-enterococcal strains (eg *S. bovis* and *S. equinus*) (Musher, 1990). Enterococci are further distinguished from other streptococci by their ability to grow between 10°C and 45°C, at pH 9.6 and in the presence of 6.5% NaCl (Holt *et al*, 1994). The genus streptococcus was eventually divided into three genera, streptococcus, enterococcus and lactococcus, such that *S. faecalis* and *S. faecium* became *Enterococcus faecalis* and *Enterococcus faecium*, respectively (Schleifer and Kilpper-Balz, 1984).

1.3. The Enterococci.

The genus *Enterococcus* includes the strains shown in table 1.1. Of these strains, only two are routinely isolated, namely *E. faecalis* (85%-90%) and *E. faecium* (5%-10%) (Moellering, 1992).

Table 1.1. Currently accepted species of enterococci (Moellering, 1992).

E. faecalis	*E. avium*
E. faecium	*E. raffinosus*
E. casseliflavus	*E. pseudoavium*
E. gallinarum	*E. malodoratus*
E. mundtii	*E. durans*
E. solitarus	*E. hirae*

Enterococci are found in the gut and faeces of virtually all healthy humans. They are less commonly isolated from the oral cavity, gallbladder, urethra and vagina. Although once thought of as harmless commensals, the frequency of enterococcal infections has increased, and enterococci are now recognised as significant pathogens (Moellering, 1992). The 1991 National Nosocomial Infection Survey of the USA named enterococci as the second most common cause of infection (behind *E. coli*, and ahead of *Pseudomonas aeruginosa* and *Staph. aureus*), responsible for 12% of nosocomial infections in the USA between 1986 and 1989 (Schaberg *et al*, 1991). The

enterococci are known to cause a number of infections, including wound infections, soft tissue infections, and rare cases of meningitis and pneumonia (Tailor *et al*, 1993). The more significant enterococcal infections are detailed below.

1.3.1. Enterococcal endocarditis.

Enterococci are the third most common cause of endocarditis, accounting for up to 5-18% of cases (Scheld and Sande, 1990; Megran, 1992). Those most at risk from enterococcal endocarditis are the elderly (mostly males over 60 years), and intravenous drug abusers (Tailor *et al*, 1993). As discussed in section 1.3.4., the most commonly identified source of bacteraemia prior to enterococcal endocarditis is the genitourinary tract (Megran, 1992). The prevalence amongst elderly men is apparently due to the increased number of genitourinary procedures performed, whilst in women, risk factors also include gynaecological procedures (Tailor *et al*, 1993).

Whilst bacteraemia is a prerequisite for endocarditis, only a small proportion of patients with significant enterococcaemia have endocarditis. Of the 153 cases of enterococcal bacteraemia reviewed by Maki and Agger (1988), only 13 developed endocarditis. Of these, all had previous heart murmur and 77% had underlying valvular heart disease. Most significantly, 12 of the 13 cases were community-acquired, and none were associated with polymicrobial bacteraemia. The explanation for this may be that community-acquired enterococcal bacteraemia evades detection longer than an equivalent nosocomial infection, allowing greater opportunity for the development of endocarditis. Conversely, polymicrobial bacteraemia is associated with high and rapid mortality such that patients may die before endocarditis is established (Maki and Agger, 1988).

Native valve enterococcal endocarditis (NVE) is most commonly a subacute infection, and presents in a manner similar to subacute endocarditis of other causes. The infection is usually left sided, even in intravenous drug abusers, affecting the mitral or aortic valves, or less commonly both together (Murray, 1990; Megran, 1992).

Aortic infection frequently requires both aggressive antibiotic therapy and surgical intervention to maximise cure rates. Estimates of the overall mortality due to NVE range from 15-40%, with higher mortality amongst patients with aortic and combined mitral and aortic valve involvement than in those with mitral valve infection alone (Rice *et al*, 1991; Almirante *et al*, 1991; Megran, 1992). In contrast, prosthetic valve enterococcal endocarditis (PVE) appears to have a relatively good prognosis. Recently, two studies were performed, comparing enterococcal infection of native and prosthetic valves. In each case, PVE responded more favourably to antibiotic therapy, and was associated with significantly lower mortality than patients with NVE. The mean duration of symptoms prior to clinical diagnoses were 17.4-18.5 days for PVE and 29.4-31.2 days for NVE. This difference reflects a heightened awareness of risk of endocarditis among patients with prosthetic valves, and may explain the difference in severity between the two forms of the disease, since a prolonged period of disease prior to diagnosis is associated with an increased incidence of relapse and death (Rice *et al*, 1991; Almirante *et al*, 1991).

1.3.2. Intraabdominal infections.

Enterococci are a major part of the normal intestinal flora, and as such are often isolated from intraabdominal abscesses, usually in combination with other organisms (Murray, 1990). Their role in the pathogenesis of such infections is unclear, however, it has been shown that enterococci can translocate from the intestinal lumen and cause abdominal infection in mice (Wells *et al*, 1990), and in another study, the presence of *E. faecalis* in polymicrobial abdominal infections appeared to be associated with increased morbidity and mortality (Matlow *et al*, 1989), thus *E. faecalis* appears to play a significant role in these infections. Furthermore, the presence of *E. faecalis* abscesses represents a significant risk factor for development of a potentially more serious bacteraemia (Graninger and Ragette, 1992).

1.3.3. Urinary tract infection.

The most common enterococcal infection is of the urinary tract, usually nosocomial and associated with instrumentation such as urethral catheterisation. *E. faecalis* strains isolated from urinary tract infections (UTIs) appear to have adhesins specific to urinary tract cells, which may allow more efficient colonisation (Guzman *et al,* 1989). Over the course of a 20 year study by Felmingham *et al,* (1992), the frequency of isolation of enterococci from UTIs in hospitals rose from 4% in 1971 to 12.6% in 1990, and the 1991 National Nosocomial Infection Survey of the USA listed enterococcus as the second most common cause of UTIs, isolated from 16% of cases (Schaberg *et al,* 1991). The apparent increase in frequency of nosocomial enterococcal urinary tract infection is likely to be linked to the increasing use of catheterisation, prior administration of antibiotics which select for resistant enterococci, and the transmission of resistant organisms by contaminated hospital equipment and personnel (Murray, 1990). Felmingham *et al,* (1992) found that urethral catheterisation preceded infection in 95% of cases. Although most cases of enterococcal UTI are asymptomatic colonisation, the presence of enterococci in the urinary tract is also a risk factor for the development of bacteraemia (Graninger and Ragette, 1992).

1.3.4. Enterococcal bacteraemia.

According to a 14 year study by Maki and Agger, (1988), enterococcal bacteraemia is most likely nosocomial (77% of cases), and usually occurs in significantly compromised patients (86%). Other risk factors include the presence of an indwelling urethral or vascular catheter (76%) and prior administration of antimicrobial therapy (63%). The most commonly identified source of infection is the genitourinary tract, being the primary infection site in 21% of cases, followed by the intraabdominal cavity and wound infections (Graninger and Ragette, 1992; Megran, 1992). The mortality of patients with enterococcal bacteraemia is 43-46% (Maki and Agger, 1988; Noskin *et al,* 1991; Graninger and Ragette, 1992), however, mortality is

significantly higher among patients with polymicrobial bacteraemia, and in cases where septic shock occurs (Noskin *et al*, 1991). It is often difficult to establish the contribution of enterococcal bacteraemia to the cause of death, since most patients have severe underlying disease, or concomitant infection by other organisms. (Maki and Agger, 1988).

1.4. Problems in therapy of enterococcal infections.

Enterococci are not considered particularly virulent organisms. Most enterococcal infections are nosocomial and opportunistic. However, enterococci are still considered significant pathogens because of their ability to resist antimicrobial agents directed against them, by both intrinsic and acquired mechanisms (Moellering, 1991; Moellering, 1992). The nosocomial nature of most enterococcal infections compounds the problem, selecting for resistant strains by the large scale use of antibiotics, and providing routes for rapid dissemination (Murray, 1990). The emergence and spread of such antibiotic resistance over the last decade has narrowed the already limited options for treatment of serious enterococcal infections such that even with the best current options, antibiotic failures are becoming commonplace (Moellering, 1992; Eliopoulos, 1993a; Swartz, 1994). Resistance may be acquired or intrinsic; the various mechanisms of enterococcal resistance to antibiotics are discussed below.

1.4.1. Intrinsic resistance.

Enterococci are intrinsically resistant to a number of antimicrobial agents, including β-lactams and aminoglycosides. β-Lactam resistance (especially to cephalosporins) is a characteristic of enterococci (Musher, 1990) and appears to be a result of the low affinity of certain enterococcal penicillin binding proteins for β-lactams (Williamson *et al*, 1985; Fontana *et al*, 1992). This resistance is present in all

strains of enterococci, including those never previously exposed to antibiotics, however, resistance is enhanced following exposure of the cells to stepwise increases in penicillin concentration, suggesting that treatment regimen may significantly affect response to β-lactam therapy (Hodges *et al*, 1992). Low-level resistance to aminoglycosides is also a characteristic of enterococci, apparently due to poor uptake through the cell wall (Moellering and Weinberg, 1971; Murray, 1990). This relatively low level resistance precludes the use of aminoglycoside monotherapy, since the required MICs exceed achievable serum levels (Musher, 1990). The lack of sensitivity to β-lactams and aminoglycosides individually has not caused clinical problems in the past because of the synergistic effect observed when these agents are used in combination. This effect is apparently due to enhanced uptake of aminoglycosides by enterococci when grown in the presence of any cell wall active drug, including β-lactams and glycopeptides (Moellering and Weinberg, 1971; Zimmerman *et al*, 1971). Enterococci also exhibit low-level intrinsic resistance to clindamycin, and to the normally synergistic combination of trimethoprim and sulfamethoxazole (Moellering, 1992).

1.4.2. Acquired resistance.

Enterococci have the ability to gain resistance to a number of antibiotics by the acquisition of exogenous DNA. Mechanisms of acquisition include inter- and intra-generic transfer of broad host-range plasmids, (eg the macrolide resistance plasmid pAMβ1, Schaberg and Zervos, 1986), high efficiency plasmid transfer via the pheromone-inducible conjugation system of *E. faecalis* (discussed in section 1.5.) and via transposons such as Tn*916* and Tn*917* (Clewell and Gawron-Burke, 1986; Schaberg and Zervos, 1986). Such mechanisms have lead to widespread enterococcal resistance to tetracyclines, macrolides and chloramphenicol, (Moellering, 1991). However, of greater significance is the emergence of high level resistance to aminoglycosides, β-lactams and glycopeptides.

1.4.3. High level aminoglycoside resistance.

Since the 1950s, treatment of serious enterococcal infections has exploited the bactericidal synergy between β-lactams and aminoglycosides, originally combining penicillin and streptomycin. However, by the 1970s, 25-50% of enterococci showed high level resistance to streptomycin and kanamycin (Swartz, 1994), preventing their use in synergy, and consequently gentamicin replaced streptomycin in the combination. Gentamicin resistant strains of enterococci were first identified in 1979 (Horodniceanu *et al*, 1979), and have since been isolated world-wide. In some centres up to 70% of strains have shown high level (MIC >2000μg/ml) resistance to gentamicin (Moellering, 1991; Swartz, 1994), and thus were also resistant to penicillin/aminoglycoside synergy. Aminoglycoside resistance may be due to chromosomal mutations which alter ribosomal binding or affect drug transport, or more commonly, due to acquired genes coding for enzymes which degrade the drugs (Moellering, 1991; Leclercq *et al*, 1992). Among the most frequently isolated enzymes are adenylyltransferases, which deactivate streptomycin and 3'-phosphotransferases, which deactivate kanamycin. Gentamicin resistance is determined by a single bifunctional 6'-acetyltransferase-2"-phosphotransferase enzyme, which effectively confers resistance to all aminoglycosides except streptomycin (Ferretti *et al*, 1986; Moellering, 1991). Thus when treating serious enterococcal infections, it is important to screen for resistance to streptomycin, kanamycin and gentamicin before beginning therapy (Leclercq *et al*, 1992)

1.4.4. High level β-lactam resistance.

To compound the problem of aminoglycoside resistance, high level resistance to β-lactams has emerged among enterococci, both by an enhancement of intrinsic resistance, especially in *E. faecium*, through increased production of low affinity penicillin binding proteins (Boyce *et al*, 1992 Chirurgi *et al*, 1992; Fontana *et al*, 1992), or by the acquisition of β-lactamase genes on plasmids (Murray *et al*, 1986)

and transposable elements which insert into the chromosome (Rice *et al*, 1991). Hybridisation and biochemical studies have suggested that the β-lactamase produced by resistant enterococci is closely related to that previously isolated from *S. aureus*, suggesting that the resistance may have originated in staphylococci and spread to enterococci (Murray *et al*, 1986a). Some success in treating these resistant organisms has been demonstrated with imipenem, or with combinations of ampicillin with the β-lactamase inhibitor sulbactam (Patterson and Zervos, 1989; Markowitz *et al*, 1991). However, many strains of β-lactam-resistant enterococci also possess high level gentamicin resistance, making infections by these organisms extremely difficult to eradicate, as illustrated by a recent report of an outbreak of infections due to β-lactamase-producing, gentamicin-resistant *E. faecalis* (Wells *et al*, 1992). Urinary tract infections are less problematic, since drug accumulation in the urine can give rise to concentrations high enough to overcome resistance mechanisms (Wells *et al*, 1992) however, bacteraemias and endocarditis remain a significant problem. Penicillin resistance due to β-lactamase production is still uncommon, however it presents a major threat, especially since conjugative transfer has been demonstrated (Coudron *et al*, 1992; Swartz, 1994).

1.4.5. Glycopeptide resistance.

Because of their reliable activity against enterococci, the glycopeptide drugs vancomycin and teicoplanin have proved useful alternatives for treatment of severe enterococcal infections in cases of penicillin allergy or β-lactam resistance (Leclercq *et al*, 1992). They are normally used in synergy with an aminoglycoside, although some reports claim 71% cure of *E. faecalis* endocarditis with teicoplanin alone (Schmit, 1992). However, the cure rate was higher in combination with an aminoglycoside. The first strains of enterococci resistant to vancomycin and teicoplanin emerged in the late 1980's (Leclercq *et al*, 1988; Uttley *et al*, 1988). Glycopeptide resistant enterococci have since been reported world-wide (Leclercq *et al*, 1992) and the rate of isolation of resistant strains appears to be increasing. The

percentage of nosocomial enterococci resistant to vancomycin in U.S.A. hospitals increased from 0.3% in 1989 to 7.9% in 1993, and the rate was higher (13.6%) among isolates from patients in intensive care units (Centres for Disease Control, 1993). The true figure may be even higher, since some glycopeptide resistant enterococci will not grow in the absence of vancomycin, and so may be missed in routine screening (Woodford *et al*, 1994). In one outbreak in an intensive care unit, vancomycin-resistant *E. faecium* were spread to numerous patients via non-disposable handles of rectal thermometers (Livornese *et al*, 1992), in another, the organisms spread from a hospital ward to a local nursing home, where patients were received after hospital discharge (Frankel, 1994). The problem is being addressed by providing clinicians with a limited list of situations where vancomycin therapy is appropriate or acceptable (Frankel, 1994) to reduce the likelihood of selection of vancomycin-resistant strains.

Glycopeptide resistance can occur through three phenotypes, VanA, VanB and VanC, although only VanA and VanB have been noted in *E. faecalis* (Arthur and Courvalin, 1993). The genes for expression of the VanA phenotype are carried upon a transposon designated Tn*1546* (Arthur *et al*, 1993), which has been found to insert both chromosomally and on plasmid DNA (Woodford *et al*, 1993). VanA enterococci are resistant to both vancomycin and teicoplanin apparently because of a modification of the target site for these drugs, namely the D-alanyl-D-alanine terminus of stem pentapeptides in bacterial peptidoglycan. Analysis of the cytoplasmic precursors of peptidoglycan in VanA strains of enterococci suggests that the D-alanyl-D-alanine moiety is altered to D-alanyl-D-lactate (Billot-Klein *et al*, 1992; Handwerger *et al*, 1992; Messer and Reynolds, 1992), resulting in reduced affinity for glycopeptides. Enterococci expressing the VanB phenotype are moderately resistant to vancomycin, but generally sensitive to teicoplanin (Arthur and Courvalin, 1993), although a recent report suggests that increasing numbers of teicoplanin resistant VanB enterococci are being isolated (Dean *et al*, 1994). The *vanB* gene cluster is transferred by chromosomal insertion of a large (90-250 kb) transposable element (Quintiliani and

Courvalin, 1994). Cloning of the *vanB* gene has revealed significant homology to *vanA* (Evers *et al*, 1993; 1994), and modified peptidoglycan precursors ending in D-ala-D-lactate, identical to those seen in VanA enterococci, have been detected in VanB strains (Billot-Klein *et al*, 1994). The lower level of resistance to glycopeptides of VanB strains, as compared to VanA strains is probably related to the presence of a pool of residual D-alanyl-D-alanine precursors detected in VanB enterococci (Billot-Klein *et al*, 1994).

1.4.6. Treatment of infections due to resistant enterococci.

The emergence of high level resistance to glycopeptides has worsened the already difficult clinical problem of therapy of serious enterococcal infections such as endocarditis. Current recommendations for the treatment of enterococcal endocarditis specify the combination of a β-lactam with an aminoglycoside for at least 4-6 weeks, with substitution of a glycopeptide where the use of β-lactams is inappropriate (Tailor *et al*, 1993). Successful treatment of a case of aminoglycoside-resistant *E. faecalis* endocarditis with high dose ampicillin has been reported (Jones, 1994), however, the susceptibility of individual strains varies. In cases where all aminoglycosides are resisted, the clinician must select the most active single drug regimen available, based on MIC reports for the isolate (Tailor *et al*, 1993).

Similarly, in cases where enterococci are resistant both to β-lactams and glycopeptides, serious problems arise, and the situation may be worsened by concomitant gentamicin resistance (Sader *et al*, 1994). In view of this real problem of increasing enterococcal resistance, the efficacy of other antibacterial combinations has been investigated. Some instances of vancomycin-ampicillin synergy in vitro have been suggested, but reports are conflicting, and susceptibility testing for individual strains is recommended (Cercenado *et al*, 1992; Eliopoulos, 1993; 1993a). High dose ciprofloxacin has been shown to be bactericidal, however its use in monotherapy is not recommended because of the high risk of selection of resistant organisms

(Bauernfeind, 1992). However, combining ciprofloxacin with gentamicin, rifampicin or both has been shown to be effective in a rat model of enterococcal endocarditis (Whitman *et al*, 1993). Evaluation of trospectomycin, a new aminocyclitol antibiotic, has shown bactericidal activity against some enterococci when used in combination with ampicillin in vitro, however, this combination is yet to be tested in vivo (Mobarakai *et al*, 1994). Another new drug, levofloxacin, has also shown bactericidal activity against multidrug resistant *E. faecalis*, when used in combination with ampicillin in vitro, however individual strain susceptibility varies (Smith and Fu, 1994).

There is concern that multiply resistant enterococci may serve as a pool of resistance genes for spread to other genera. Of particular concern is the ability of enterococci to transfer high level vancomycin resistance to staphylococci (Noble *et al*, 1992), raising the possibility of glycopeptide resistance in *S. aureus*. If resistance to currently available antibiotics continues to emerge, then effective treatment of enterococcal infections may require the development of a new generation of antimicrobial drugs (Eliopoulos, 1993a). Thus a detailed understanding of the pathogenic mechanisms of enterococci is essential. Despite their being the third most common aetiologic agents in infective endocarditis, little is known of the pathogenic mechanisms of enterococci in this respect, with most research having been directed at viridans streptococci and staphylococci.

1.5. Pheromone-induced plasmid transfer in *E. faecalis*.

A feature apparently unique to enterococci is the ability to acquire conjugative plasmids at very high efficiency (enhanced by 10^5-10^6-fold for broth matings), in response to sex pheromones released by plasmid-free strains (Clewell, 1993).

Early evidence of the ability of *E. faecalis* to transfer resistance plasmids by conjugation was provided by Jacob and Hobbs (1974), who later showed that haemolysin determinants transferred in a similar manner (Jacob *et al*, 1975). Since then, the pheromone-inducible plasmid transfer system of *E. faecalis* has been well characterised (Clewell, 1993; Wirth, 1994). *E. faecalis* strains harbouring sex pheromone plasmids (donor cells) clump when mixed with cells not containing that particular plasmid (recipient cells), whereas both strains show normal behaviour when incubated alone (Dunny *et al*, 1978). During this clumping stage, plasmid transfer occurs from donors to recipients. Once transfer is complete, the clumps disperse, the population having shifted to one almost entirely of donors (Wirth, 1994). Some of the *E. faecalis* sex pheromone plasmids carry bacteriocin/haemolysin determinants, which have been shown to contribute to virulence (Ike *et al*, 1984; Jett *et al*, 1992; Chow *et al*, 1993), others encode antibiotic resistance (Murray *et al*, 1988).

At least 19 pheromone-responsive plasmids are known to exist, each one responding to a particular pheromone (Wirth, 1994). The sex pheromones are hepta- or octa-peptides, secreted by recipient strains, indicating to donors that they do not possess the corresponding plasmid (Clewell and Weaver, 1989). When donor cells sense the corresponding pheromone, a plasmid-encoded aggregation substance (AS) is synthesised, which mediates bacterial clumping by adhesion to a chromosomally-encoded binding substance (BS) present on all other *E. faecalis* strains (Clewell and Weaver, 1989). Since donor cells also express BS, they bind to each other as well as to recipient strains (Dunny *et al*, 1978). Free lipoteichoic acid was found to inhibit the clumping response, suggesting that cell surface LTA may represent BS (Ehrenfield *et al*, 1986). However, more recent mutagenesis studies suggest that the situation is more complex, involving surface proteins as well as LTA (Bensing and Dunny, 1993).

Aggregation allows plasmid transfer events to occur, however the adhesion is independent of the transfer process, which requires other plasmid directed proteins (Olmsted *et al*, 1991). During aggregation, donor cells produce a surface exclusion

protein, which prevents transfer of plasmids from donor to donor (Weidlich *et al*, 1992), increasing the efficiency of conjugation. When a cell acquires a certain plasmid, it shuts off production of the inducing pheromone, and initiates production of a pheromone inhibitor, to prevent auto-aggregation in response to any residual pheromone production (Clewell, 1993). Different sex pheromone plasmids operate independently of each other, since when an acquired plasmid directs shut off secretion of the corresponding pheromone, other pheromones are unaffected. Furthermore, when a donor containing multiple plasmids is induced by a single pheromone, only the corresponding plasmid is transferred (Ehrenfield, 1986).

1.5.1. The dual role of aggregation substance.

The AS produced by donor cells in response to pheromone has been visualised on the cell surface as a dense mat of hair-like structures (Galli *et al*, 1989; Wanner *et al*, 1989) directly involved in cell-cell interactions (Olmsted *et al*, 1993). The aggregation substances of all known sex pheromone plasmids, with one exception, are homologous (Galli and Wirth, 1991), and have been shown to include the RGD motifs Arg-Gly-Asp-Ser, and Arg-Gly-Asp-Val (Galli *et al*, 1990; Kao *et al*, 1991), which are found in proteins mediating adhesion to eukaryotic cells via specific receptors (Ruoslahti, 1991). This finding led to the hypothesis that AS might mediate attachment to host cells, and thus act as a virulence factor. This was confirmed by Kreft *et al*, (1992) who not only demonstrated *E. faecalis* attachment to eukaryotic cells via the RGD motifs, but also found that synthesis of AS was induced by some component(s) of serum. This suggests that *E. faecalis* may be able to sense transition into the host, and respond by colonising tissues through expression of AS (Wirth, 1994). Aggregation substance also appears to have a role in the virulence of *E. faecalis* in endocarditis, contributing to the size of vegetations, but not to infectivity (Chow, *et al*, 1993).

1.6. Other organisms in infective endocarditis.

A list of aetiologic agents in endocarditis is shown in table 1.2. The pathogenesis of infective endocarditis is presumed to start with the adherence to, and colonisation of cardiac tissues by organisms which gain access to the circulation during brief bacteraemias. The relatively narrow spectrum of organisms which routinely cause endocarditis, as compared to the large number of organisms frequently isolated from blood cultures suggests that the events leading to the colonisation of the heart valve are not random (Johnson, 1993). It is probable that the difference in ability of organisms to cause endocarditis rests, at least in part, with their ability to adhere to cardiac tissues (Barco, 1991). The mechanisms by which these events occur are discussed in section 1.12.

Table 1.2. Aetiologic agents in infective endocarditis. Figures indicate percent of cases.

Causative agent.	Scheld and Sande, 1990	Watanakunakorn and Burkert, 1993
Streptococci	60-80%	44%
Viridans streptococci	30-40%	35%
Enterococci	5-18%	8%
Other streptococci	15-25%	8.5%
Staphylococci	20-35%	27%
Coagulase-positive	10-27%	20%
Coagulase-negative	1-3%	7%
Gram -ve aerobic bacilli	1.5-13%	
Fungi	2-4%	
Misc. Bacteria	<5%	
Mixed infections	1-2%	
Culture negative	<5-24%	

1.7. Antimicrobial therapy of endocarditis.

1.7.1. General considerations.

Even when treating endocarditis due to organisms which are highly sensitive to antimicrobials in vitro, complete eradication requires antimicrobial chemotherapy of up to 6-8 weeks, and relapse is not uncommon. This apparent anomaly reflects the difficulty in delivery of antimicrobial agents to the infected platelet-fibrin vegetation (Scheld and Sande, 1990). Bacteria within the endocardial vegetation are separated from plasma by layers of fibrin and platelets, and the drug levels achieved within the vegetation are inferior to those measured in plasma. Furthermore the large numbers of bacteria within the vegetation and their relative metabolic inactivity contribute to the difficulty of eradicating such infections (Carbon *et al*, 1993). This is particularly true for β-lactam antibiotics, which require cell growth in order to be bactericidal. Furthermore, with some organisms, large concentrations of β-lactamase can occur within the vegetation, which can inactivate β-lactams, even when used in combination with β-lactamase inhibitors (Carbon *et al*, 1993).

1.7.2. Choice of drugs.

In general, parenteral therapy is preferred to oral therapy, because of more controllable and reliable plasma levels, and extended drug administration is required to reduce the chances of relapse. Bacteriostatic drugs, such as tetracyclines and erythromycin should be avoided in favour of antibiotic combinations which produce a rapid bactericidal effect, such as the combination of β-lactam with aminoglycoside (Scheld and Sande, 1990). For appropriate therapy, the causative organism must be isolated before the initiation of antibiotic treatment, and its susceptibility to antibiotics determined. In the case of β-lactam resistance, vancomycin can be substituted, and in the case of streptomycin resistance, gentamicin is often effective (Eliopoulos, 1993). A typical therapeutic regimen for treating streptococcal endocarditis may consist of aqueous penicillin G, 10-20 million units daily, (by intravenous infusion) every six

hours for four weeks, combined with streptomycin, 500mg (or gentamicin 80mg) intramuscularly every 12 hours for the first two weeks (Scheld and Sande, 1990). Alternatively, the final two weeks of therapy may be by way of oral amoxycillin (Littler and Shanson, 1989).

When treating penicillin-resistant streptococci or enterococci, which are intrinsically resistant to many commonly used drugs, treatment should be in combination with aminoglycosides for 4-6 weeks, with monitoring for aminoglycoside toxicity (Scheld and Sande, 1990).

Studies on the treatment of *S. aureus* endocarditis with combined β-lactam and aminoglycoside regimens indicate that an enhanced effect is not evident after 5 days of treatment, beyond which the risk of nephrotoxicity outweighs the clinical benefits which may be achieved through continued use of these drugs (Bayer, 1993).

1.7.3. Surgery.

Cardiac surgery, particularly valve replacement has considerably reduced mortality in infective endocarditis in patients with serious haemodynamic or infective complications and where medical treatment alone is insufficient to control the disease (Littler and Shanson, 1989).

1.8. Prophylaxis of infective endocarditis.

1.8.1. General principles.

Any surgical or dental procedure which damages a mucosal surface can cause a bacteraemia which may last in excess of 15 minutes. During this time, bacteria may lodge upon the myocardium, resulting in infection. Certain cardiac conditions predispose to endocarditis, and furthermore certain dental and surgical procedures cause bacteraemia with organisms more likely to cause endocarditis than other procedures.

Thus, prophylactic therapy is recommended for those at risk of endocarditis, who are undergoing procedures likely to produce bacteraemia with endocarditis-causing organisms, such as dental procedures and genito-urinary tract or gastro-intestinal surgery (Dajani *et al*, 1990).

1.8.2. Antibiotic prophylaxis.

The aim of antibiotic prophylaxis is to provide adequate serum antibiotic levels during and after the risk procedure. Only in cases of prolonged bleeding should the treatment be continued beyond the procedure itself, to avoid the likelihood of resistance emerging. The prophylactic therapy is directed towards those organisms most likely to cause endocarditis from a given site. Thus, prophylaxis prior to dental procedures is aimed at viridans streptococci, whilst that prior to gastro-intestinal or genito-urinary tract surgery is directed towards enterococci. The recommended prophylactic regimen for dental procedures is either amoxycillin 3g, erythromycin 1g or clindamycin 300mg orally prior to procedure, followed by half the initial dose 6 hours later. If the oral route is not acceptable, ampicillin or clindamycin may be given parenterally. For genitourinary or gastrointestinal procedures, ampicillin 2g, plus gentamicin 1.5 mg/kg should be given intravenously 20 minutes before the procedure, followed by amoxycillin 1.5g, 6 hours later. In the case of penicillin allergy, vancomycin 1g (intravenous) may be substituted for ampicillin (Dajani *et al*, 1990).

Because of the prevalence of endocarditis-causing viridans streptococci in the oral cavity, patients undergoing dental procedures are particularly at risk. Accordingly, the American Heart Association recommends that risk patients should establish and maintain the best possible oral health. Furthermore, the application of chlorhexidine mouthwash prior to dental procedures as an adjunct to antibiotic prophylaxis is suggested (Dajani *et al*, 1990).

The antibiotic prophylaxis of endocarditis has been the subject of some controversy. A proportion of patients develop endocarditis, even if they have no

known predisposing heart lesion, and so are not targeted for therapy, and there is no direct clinical trial evidence to support the effectiveness of antibiotic prophylaxis (Littler and Shanson, 1989). Indeed, such a trial would encounter major ethical issues. The rationale behind antibiotic prophylaxis is sound, but the fact that the number of cases of endocarditis has not dropped since the introduction of systemic antibiotics, and that some cases of endocarditis have still occurred despite appropriate prophylaxis raises questions over the efficacy of such treatments (Barco, 1991). Antibiotics do not prevent transient bacteraemia following surgical procedures, but rather they serve to eradicate the organisms once in the bloodstream, and because the sensitivity of bacteria to antibiotics can be variable (Barco, 1991), treatment with a single antibiotic may be inappropriate. However, without sound clinical evidence that such treatments are effective, it would be inappropriate to subject a patient to the risk of toxicity associated with more aggressive therapy.

1.9. Pathology of infective endocarditis.

If left untreated, the infection causes severe damage to the heart valves and surrounding tissue, can cause constant bacteraemia by release of infecting organisms into the circulation, and can lead to other systemic complications. Bacteraemia produces symptoms of fever, rigors, anaemia and anorexia (Littler and Shanson, 1989). Activation of host defence mechanisms leads to the presence of circulating immune complexes, which can become lodged in the vasculature (Kaye, 1985), leading to a range of systemic events including renal dysfunction, splenic infarction and cerebral haemorrhage (Watanakunakorn and Burkert, 1993). During active infective endocarditis, renal architecture is always altered, even in the absence of clinical or biochemical evidence of renal dysfunction (Scheld and Sande, 1990).

1.9.1. Cardiac damage.

Local invasion of cardiac tissue leads to valve damage, often resulting in incompetence, and sometimes perforation, presenting as congestive heart failure in up to half of endocarditis cases (Megran, 1992). Perivalvular extension of infection (PVEI), ie the spread of infection from the heart valve to the surrounding tissues is a major, potentially fatal complication of endocarditis. Prosthetic valves are particularly at risk. Extension of infection most commonly occurs into the cardiac tissue immediately adjacent to the valve ring, causing perivalvular abscess, aneurysms, intracardiac fistulas and valve dehiscence. Furthermore, extension into conduction fibres can cause partial or complete heart block (Carpenter, 1991).

1.9.2. Embolic phenomena.

Sections of an infected cardiac vegetation can be dislodged from the site of infection and be carried in the circulation, leading to the formation of septic emboli and abscesses. Embolic phenomena are common in infective endocarditis, being detected in 45-65% of cases at autopsy, most commonly affecting the renal, splenic, coronary or cerebral circulation. Renal defects are invariably present, cerebral emboli occur in at least one third of all cases, and in cases where the right side of the heart is involved, pulmonary emboli are common (Scheld and Sande, 1990). If abscesses form, they can serve as sources for re-infection of the heart valve, leading to failure of therapy. If left untreated, the infection is almost invariably fatal.

1.10. Clinical presentation.

Infective endocarditis is a systemic disease which can present with unusual or important symptoms in virtually every organ system of the body. However, according to two recent surveys of endocarditis cases, the most common clinical manifestations of infective endocarditis are fever and heart murmur (Megran, 1992; Watanakunakorn and Burkert, 1993). The other symptoms and physical findings of infective endocarditis are listed according to frequency in table 1.3. The presentation of endocarditis is varied and in some cases, the presenting symptoms are so vague that it is not possible to establish infective endocarditis until autopsy (Watanakunakorn and Burkert, 1993).

Many of the clinical symptoms listed in table 1.3. are the result of peripheral embolisation or the presence of circulating immune complexes (Maisch, 1989). Ostlers nodes are small painful lesions found in the pads of fingers or toes, and arise from immune complexes in dermal blood vessels. Roth spots are retinal lesions surrounded by oedema and haemorrhage, splinter haemorrhages are seen in the finger or toe nails, and petechiae, resulting from local vasculitis or emboli usually occur on the conjunctiva or buccal mucosa. Janeway lesions, subcutaneous abscesses due to septic emboli, are relatively uncommon in endocarditis (Megran, 1992). The presentation of infective endocarditis depends very much upon the course of the infection. Enterococcal endocarditis generally presents in the subacute form, allowing many of the above symptoms to occur. Infection by *S. aureus* however is more often acute and fulminant, presenting with fever and sepsis symptoms (Bayer, 1993)

Table 1.3. Clinical features and manifestations of infective endocarditis (adapted from Scheld and Sande, 1990; Megran, 1992).

Symptom	% of cases	Clinical Findings	% of cases
Fever	80-100	ESR > 30 mm/hr	90-100
Chills	40	Heart Murmur	85-100
Weakness	40	Embolic phenomena	>50
Dyspnoea	40	Skin manifestations	18-50
Sweats	25	Ostler nodes	10-23
Anorexia/weight loss	25	Splinter haemorrhages	15
Malaise	25	Petechiae	20-40
Cough	25	Janeway lesions	<10
Skin lesions	20	Splenomegaly	20-68
Stroke	20	Septic emboli	20
Nausea/vomiting	20	Mycotic aneurysms	20
Headache	15	Clubbing	12-52
Myalgia/arthralgia	15	Roth spots	2-10
Oedema	15	Renal dysfunction	10-50
Chest Pain	15	Leukocytosis	20-30
Abdominal pain	10-15		
Delirium coma	10		
Haemoptysis	10		
Back Pain	10		

1.11. Diagnosis.

The clinical and aetiological diagnosis of infective endocarditis is of paramount importance for optimal treatment, since rational antimicrobial therapy can only be applied once the infecting organism has been identified, and its susceptibility to antibiotics determined. In its full blown state, clinical diagnosis of endocarditis is straight forward, especially when the classic manifestations such as emboli, heart murmurs and immunologic phenomena, along with positive blood cultures, are present (Bayer, 1993). However, these signs are not always displayed, especially in acute cases, and many of the symptoms of endocarditis are non-specific (Lukes *et al*, 1993).

A major problem is that the primary lesion in endocarditis is inaccessible, with open heart surgery or autopsy required for direct examination. Thus the clinician must be aware of the risk groups for endocarditis, and of the non-specific nature of presentation. Recently, a set of guidelines has been proposed, based upon major and minor diagnostic criteria (Bayer *et al*, 1994) which have greatly improved the accuracy of diagnosis of endocarditis.

1.11.1. Standard diagnostic procedures.

Transoesophageal echocardiography can identify and characterise up to 90% of cardiac vegetations (Bayer, 1993), and is recommended for all patients with suspected endocarditis (Lukes and Durack, 1993). Echocardiographic findings are not diagnostic alone, and so blood cultures should be taken as soon as infective endocarditis is suspected, before initiation of antibiotic therapy. Most cases of endocarditis are blood culture positive, however, up to 30% of cases can be culture negative (Tunkel and Kaye, 1992). This is usually due to prior antibiotic therapy, but other reasons for negative blood cultures include incorrect diagnosis, and infection by organisms which are difficult to subculture, such as members of the HACEK (*Haemophilus, Actinobacillus, Cardiobacterium, Eikenella and Kingella* sp.) group, and some nutritionally variant streptococci (Tunkel and Kaye, 1992).

1.11.2. Alternative diagnostic methods.

An alternative to aetiological diagnosis of endocarditis by blood culture is serodiagnosis, which relies upon detection of host antibodies to specific bacterial surface components. Whereas blood culture results can take over 24 hours, and in some cases may be negative, serodiagnosis is rapid, and can often distinguish between bacteraemia and endocarditis for a particular organism. Burnie and Clark, (1989) reported the use of a 112 kDa *E. faecalis* antigen in the diagnosis of enterococcal endocarditis. The test was capable of distinguishing *E. faecalis* endocarditis from that caused by staphylococci or other streptococci. Shorrock *et al*, (1990) subsequently

reported the use of three antigens, 37, 40 and 73 kDa in an ELISA which not only distinguished between *E. faecalis* and that caused by other streptococci, but also between *E. faecalis* endocarditis and other *E. faecalis* infections, such as urinary tract infections, intra-peritoneal infections and septicaemia. Serodiagnosis of *S. aureus* endocarditis among patients with *S. aureus* septicaemia, by detection of antibodies against capsular polysaccharide has been reported (Christensson *et al*, 1991).

1.12. Pathogenesis of infective endocarditis.

The establishment of a bacterial infection on the endocardium requires certain pre-disposing factors. These factors include the susceptibility of the heart valve to infection, the presence of a bacterial population capable of colonising the valve, the adhesion of bacteria to the valve and finally the survival of the adhering bacteria, with propagation of the endocardial vegetation (Sullam *et al*, 1985).

1.12.1. Non-bacterial thrombotic endocarditis.

It is generally accepted that normal healthy heart valves are refractory to infection, and that some form of pre-existing damage to the heart valve is required prior to infection (Sullam *et al*, 1985; Rice *et al*, 1991; Baddour, 1994). In animal models of endocarditis, it is well established that the formation of bacteraemia by intravenous injection of pathogenic bacteria is insufficient to cause endocarditis, and that some other form of trauma, usually in the form of a catheter across the heart valve, is required in order to produce endocarditis (Garrison and Freedman, 1970; Gutschik, 1993; Baddour, 1994). Examination of infected vegetations revealed that endocardial trauma in the form of microscopic thrombi served as the nidus for initiation of endocardial infection. In the presence of these thrombi, since termed non-bacterial thrombotic endocarditis (NBTE), bacteraemia uniformly produced infective endocarditis (Garrison and Freedman, 1970).

Normal cardiac endothelial cells are not thrombogenic, so the formation of a non-bacterial thrombotic endocarditis must follow damage to the endothelium. An accepted theory for the formation of the NBTE (Hamill, 1987), begins with some form of endothelial damage which traumatises or removes endothelial cells, exposing components of the extracellular matrix such as collagen, laminin and fibronectin. Circulating platelets become lodged in the area of damage and aggregate along with fibrin and fibronectin, which become interwoven with the adherent platelets. The vegetation (NBTE) grows as fibrin and platelets continue to bind. Circulating bacteria adhere to the NBTE, and become coated with more fibrous material and bacteria. The role of fibronectin in the formation of NBTE is significant, and will be discussed below. It is becoming increasingly apparent that numerous bacteria, whilst unable to colonise healthy endocardium, can exploit the wound healing process at the site of NBTE by interacting with various host tissues such as fibronectin (Hamill, 1987), platelets (Herzberg *et al*, 1992; Hermann *et al*, 1993), and laminin (Sommer *et al*, 1992).

1.12.2. Risk factors.

As discussed in section 1.12.1., healthy heart valves are refractory to infection; the formation of an NBTE is required prior to infection (Rice *et al*, 1991; Steckelberg and Wilson, 1993; Baddour, 1994). Endothelial trauma, inducing formation of an NBTE, and bacteraemia are the two major risk factors in infective endocarditis. A list of conditions predisposing to such trauma is shown in table 1.4.

Damage may result from previous rheumatic heart disease, where an auto immune response is directed toward heart tissue elicited by group A streptococcal antigens (Kil *et al*, 1994), or from congenital heart defects, which produce abnormally turbulent blood flow in the vicinity of the heart valve (Sullam *et al*, 1985; Fowler and Durack, 1994). The predominant underlying condition in endocarditis has shifted away from rheumatic heart disease, towards mitral valve prolapse, infection of prosthetic

Table 1.4. Risk factors in endocarditis (Littler and Shanson, 1989; Dajani *et al*, 1990)

Cardiac Factors	Extracardiac Factors
Rheumatic heart disease (15-40%)	Dental sepsis and manipulations
Congenital heart disease (15-20%)	Genitourinary/gastrointestinal surgery
Atherosclerotic heart disease (20-30%)	Intravenous drug abuse
Prosthetic valve,	Intravenous cannulae
or other cardiac surgery (10-35%)	Haemodialysis
Mitral valve prolapse	Septicaemia (from any site)
Hypertrophic obstructive cardiomyopthy	Endoscopy
Previous Endocarditis	Tonsillectomy and/or adrenoidectomy.

heart valves, degenerative heart disease and damage caused by intravenous drug abuse (Bayer, 1993). This shift is due in part to the decreased incidence of rheumatic heart disease, and partly to increased life expectancy and the frequency with which major surgery is performed (Bayer, 1993). There is also an increased incidence of neonatal endocarditis, associated with the use of central venous catheters to deliver total parenteral nutrition to premature infants of extreme low birth weight (Mecrow and Ladusans, 1994).

Some studies maintain that infection can occur on normal valves as well as those with pre-existing damage (Megran, 1992; Steckelberg and Wilson, 1993). Indeed in the study of enterococcal endocarditis by Rice *et al*, (1991), 7 of 21 patients (33%) had no apparent predisposing valvular heart disease. Furthermore, in the study by Watanakunakorn and Burkert, (1993), the figure was 46%. It is likely, however, that in these cases, some valvular damage was present, which evaded detection by conventional means.

1.12.3. Bacteraemia.

Bacteria can enter the blood stream from many sites, often when a mucosal surface, colonised with bacteria, becomes traumatised. The most likely portal of infection depends upon the infecting organism. α-Haemolytic streptococci (including *S. sanguis*, *S, gordonii* and *S. mitis*), are present in high numbers in the oral cavity.

These bacteria can enter the blood stream through breaks in the blood vessels of oral tissues caused by dental procedures such as extractions, oral infections and oral hygiene procedures. In a study of rats with periodontitis and catheter-induced aortic vegetations, all animals which underwent dental extraction displayed polymicrobial bacteraemia one minute later, and within three days 90% had endocarditis (Moreillon *et al*, 1988). A 13-year survey of enterococcal bacteraemia lists the most common sources of infection as the gastro-intestinal tract (following surgery or intraabdominal infection), the urinary tract (also following surgical intervention, such as urethral catheterisation), burn wounds and vascular catheter-related infections (Maki and Agger, 1988). Staphylococcal endocarditis is common following heart surgery, *S. aureus* and *S. epidermidis* being the predominant organisms in prosthetic valve endocarditis. The source of infection may be the prosthesis itself, contaminated surgical equipment, or post operative wound infection (Littler and Shanson, 1989). Intravenous drug abuse is also a common source of *S. aureus* bacteraemia (Bayer, 1993, Lukes and Durack, 1993).

1.12.4. The role of platelets in the pathogenesis of infective endocarditis.

Platelets play a central role in the pathogenesis of endocarditis. It is uncertain, however, if platelets serve to enhance or limit propagation of the infected vegetation. A substantial proportion of the NBTE, the focus of bacterial infection, consists of aggregated platelets, and endocarditis models using animals with clotting disorders have shown that normal platelet function is an important determinant in the pathogenesis of the disease (Johnson and Bowie, 1992). Certain strains of *S. sanguis*, have been shown to bind to, and activate, platelets via protein determinants on the bacterial cell surface (Herzberg *et al*, 1983; 1983a). The platelet interaction is mediated via at least two *S. sanguis* fibrillar antigens, one of which mediates adherence to platelets, the other triggering aggregation (Erickson and Herzberg, 1993; Ford *et al*, 1993). Aggregation appears to be mediated via interaction with plasma

components, rather than by direct association with platelet receptors (Ford *et al*, 1993a).

In a clinical study, 70% of severe cases of infective endocarditis were caused by platelet-aggregating streptococci, compared to 14% of mild cases (Greaves *et al*, 1993). In a rabbit model, *S. sanguis* strains which expressed platelet aggregation-associated protein (PAAP) (Agg^+ strains) caused endocarditis with significantly larger vegetations, a more severe clinical course and greater mortality than mutants deficient in PAAP expression (Agg^- strains), or Agg^+ strains incubated with anti-PAAP antibody (Herzberg *et al*, 1992). The investigators thus proposed that the PAAP was an important virulence factor in infective endocarditis. Platelets have also been proposed as important mediators of *S. aureus* adhesion in endocarditis and device-associated infections. Adherence of *S. aureus* to inert supports was shown to be enhanced by the presence of adherent platelets, along with other plasma proteins, particularly fibronectin (Herrmann *et al*, 1993).

In contrast to the studies detailed above, a study by Sullam *et al*, (1993), concluded that platelets limit the progression of streptococcal infective endocarditis. In this study, the role of platelets was analysed by comparing *S. sanguis* endocarditis in normal and thrombocytopaenic rabbits (in which thrombocytopaenia was induced with antiplatelet serum). Vegetations from thrombocytopaenic rabbits weighed significantly less than those from normal rabbits, but contained more bacteria, suggesting that platelets in the vegetation can limit microbial proliferation. The authors suggest that platelets may attenuate disease progression by release of platelet microbicidal proteins in the vegetation. During clotting, platelets release antibacterial proteins which can kill or exert non-lethal antiadherence affects on Gram-positive organisms including some strains of viridans streptococci and staphylococci (Yeaman *et al*, 1994). Resistance to such antibacterial agents may represent a virulence factor in infective endocarditis (Yeaman *et al*, 1992). Platelets may similarly interact with bloodborne organisms, reducing the number of bacteria available to colonise the vegetation (Sullam *et al*,

1993). Thus the role of platelets in the pathogenesis is far from clear. They appear to be vital for the pre-disposition to infection, but subsequently may reduce the severity of endocarditis.

1.12.5. The role of fibronectin in the pathogenesis of endocarditis.

Fibronectin, an adhesive glycoprotein, is a major component of the extracellular matrix, where it serves to promote adhesion of cells and other matrix macromolecules, having binding sites for eukaryotic cells, collagen and other matrix components (Alberts *et al*, 1989). Fibronectin can exist as plasma fibronectin, a circulating soluble dimeric form or as matrix fibronectin, highly insoluble fibrils in the extracellular matrix. It is not present on the lumenal surface of endothelial cells, however at sites of endothelial damage it is exposed, and promotes thrombogenicity of the site of injury by binding platelets and fibrin. Plasma fibronectin further enhances clot formation by binding to the NBTE, ultimately constituting 4.4% of the blood clot mass (Hamill, 1987). Thus fibronectin has a substantial role in formation of sterile vegetations. However, of equal importance is the capacity of micro-organisms to bind specifically to fibronectin.

1.12.6. Bacterial adhesion to fibronectin.

Many bacteria which are known to cause endocarditis, can bind fibronectin (Baddour, 1994). The ability of *S. sanguis* and *S. aureus* to bind to fibronectin-coated surfaces has been demonstrated (Lowrance *et al*, 1988; Herrmann *et al*, 1988; Vaudaux *et al*, 1992). These organisms are amongst the most common endocarditis pathogens, and their ability to bind fibronectin is considered central to their pathogenesis. It would appear that *S. sanguis* selectively binds immobilised fibronectin, rather than soluble fibronectin (Lowrance *et al*, 1988), a property which may result from an activation of fibronectin when it becomes bound to other molecules or substrates. The importance of fibronectin binding in *S. sanguis* endocarditis has been demonstrated by Lowrance *et al*, (1990), who compared the

virulence of non-fibronectin-binding *S. sanguis* mutants with that of the parent strain. In a rat model, non-fibronectin-binding mutants rarely produced endocarditis, whilst parent strains had infection rates of 90% or higher, with a low inoculum. Similarly in *S. aureus*, mutants defective in production of certain cell surface proteins involved in adhesion to fibronectin and fibrinogen had reduced virulence in an endocarditis model (Cheung *et al*, 1994). Thus, fibronectin has a significant role in the pathogenesis of endocarditis. Bacterial fibronectin binding proteins are discussed in section 4.5.18.

1.12.7. Bacterial adhesion to other extracellular matrix components.

The ability of endocarditis-associated streptococci to bind to other matrix components exposed at the damaged endocardium has been reported. Laminin binding is common among endocarditis isolates, but less common among the same bacterial species isolated from the oral cavity (Switalski *et al*, 1987). Laminin binding proteins of *S. gordonii* have been identified, and appear to be inducible by components of the extracellular matrix (Sommer *et al*, 1992). Furthermore, *S. pyogenes* appears to express proteins with affinity for connective tissue proteoglycans in basal laminae of cardiac muscle and other tissues (Winters *et al*, 1993). The relative importance of such interactions is difficult to establish, since even for major binding processes, such as fibronectin binding, eliminating the binding ability does not completely abrogate the ability of the organism to produce infection (Lowrance *et al*, 1990; Cheung *et al*, 1994). Thus the pathogenesis of infective endocarditis is likely to be multifactorial and may involve many as yet uncharacterised ligand receptor interactions, as well as other types of virulence factors.

1.12.8. Glycocalyx production.

The platelet interactive proteins and extracellular matrix binding proteins of Gram-positive organisms described above are virulence factors in endocarditis. There

are reports of several other bacterial cell products which appear to enhance the ability of the bacterium to cause endocarditis, and so may be classified along with the extracellular matrix-, and platelet-binding proteins as virulence factors. Of particular interest is the presence of an extracellular polysaccharide coat (glycocalyx) on certain endocarditis-causing bacteria. An association between surface glucan production and the ability of streptococci to bind to heart valves in vitro is well established (Scheld *et al*, 1978; Ramirez-Ronda, 1978), and the presence of a glycocalyx has since been considered a virulence factor in endocarditis. Viridans streptococci, including *S. sanguis* and *S. mutans*, produce exopolysaccharide. A comparison of glycocalyx production by endocarditis and non-endocarditis streptococci by Dall and Herndon, (1990), suggested an association between expression of a glycocalyx and the ability of viridans streptococci to cause endocarditis. This was confirmed by Munro and Macrina (1993), who demonstrated that exopolysaccharide production by *S. mutans* V403 was associated with an enhanced ability to cause endocarditis in a rat model, and resistance to phagocytosis. The production of exopolysaccharide appeared to enhance adherence to fibrin, which may provide an explanation for the enhanced virulence of these strains. However, in a similar study using *S. gordonii*, the virulence of mutants defective in exopolymer production was no different to that of the parent strain (Wells *et al*, 1993). Indeed, the effect of glycocalyx appears to vary according to the organism concerned. In a rat model, *S. aureus* strains lacking a microcapsule produced endocarditis at lower inocula than encapsulated strains, suggesting that capsule expression, rather than being a virulence factor, actually reduces virulence (Baddour *et al*, 1992). Thus, generalisations about the contribution of exopolysaccharide to virulence of viridans streptococci in endocarditis appear to be inappropriate. A recent report by Vacca-Smith *et al*, (1994), may provide some explanation of the disparate findings of the studies described above. The investigators found that a glucosyltransferase (GTF) enzyme, of the type responsible for production of exopolysaccharide, mediated adhesion of *S. gordonii* to human umbilical vein endothelial cells. This is consistent with the association between exopolysaccharide

production and virulence in endocarditis (Dall and Herndon, 1990). It was this type of enzyme which was inactivated in order to produce exopolysaccharide-deficient mutants in the studies by Munro and Macrina, (1993) and Wells *et al*, (1993). Vacca-Smith *et al*, (1994), reported that both bacterial adhesion and glucosyltransferase enzyme activity decreased rapidly during stationary phase growth, thus GTF mutants may behave similarly to stationary phase parent cells. Furthermore, it is not known whether the cells inoculated by Wells *et al*, (1993), adhered to endothelial cells, (the target for adhesion of the GTF enzyme), or pre-existing catheter induced thrombi.

The virulence-enhancing mechanism of the glycocalyx has mainly been attributed to its ability to enhance binding to platelet-fibrin vegetations, and to produce larger vegetations, restricting susceptibility to antimicrobial agents (Dall and Herndon, 1989; Dall *et al*, 1990). However, Sullam *et al*, (1993a), found that streptococcal glycocalyx production correlated with reduced binding to platelets, and that exopolysaccharide itself could inhibit platelet aggregation. In the light of previous findings that platelet interactions may be detrimental to the invading pathogens (Yeaman *et al*, 1992; 1994; Sullam *et al*, 1993), the authors postulated that this avoidance of platelets may represent an additional mechanism by which the glycocalyx can contribute to virulence in endocarditis. Further studies may be needed to clarify the relative importance of exopolysaccharide production and GTF activity in the pathogenesis of infective endocarditis.

1.12.9. Autolysin production.

The production of autolytic enzymes by Gram-positive organisms has been linked to virulence. Autolytic enzymes appear to degrade the peptidoglycan cell wall, possibly functioning in wall growth, cell separation, wall turnover, and extrusion of flagella. Their role in pathogenicity has been demonstrated in *S. pneumoniae*, where autolysin-defective mutants were less virulent than parent strains. Furthermore, immunisation with purified autolysin protected mice from *S. pneumoniae* challenge

(Berry *et al*, 1989). Autolysin-defective mutants of *S. aureus* have been isolated (Mani *et al*, 1993), and compared with a parent strain in a rat model of endocarditis. Six of nine rats challenged with the parent strain developed endocarditis, whereas none of the eight inoculated with autolysin-defective mutants were infected (Mani *et al*, 1994). The reduced virulence of the mutants may have been related to alterations in cell wall morphology, as detected by electron microscopy, rather than any direct effect upon the host.

1.13. Pathogenesis of *E. faecalis* in endocarditis.

Early work by Gould *et al* (1975) showed that *E. faecalis* bound to heart valve tissue more avidly than most other streptococci and staphylococci, suggesting that the species may possess specific binding determinants. Guzman *et al*, (1989), demonstrated that the ability of *E. faecalis* to adhere to heart cells was dependent upon its growth medium, and later showed that enhanced adhesion was due to serum-dependent expression of D-galactose and L-fucose containing moieties (Guzman *et al*, 1991b). Growth in serum was also associated with decreased hydrophobicity, which may be responsible for reduced association with human polymorphonuclear leukocytes noted in serum-grown strains of *E. faecalis* (Guzman *et al*, 1991a).

Enterococcus faecalis strains bearing certain pheromone-responsive plasmids, such as pAD1, express a haemolysin/bacteriocin protein, also referred to as cytolysin, which has been reported to contribute to virulence in intraperitoneal infections of mice (Ike *et al*, 1984), human parenteral infections (Ike *et al*, 1987), and experimental endophthalmitis (Jett *et al*, 1992). *E. faecalis* strains bearing these plasmids also express aggregation substance. The role of the plasmid-associated haemolysin and aggregation substance, both separately, and in combination in *E. faecalis* endocarditis has been investigated (Chow *et al*, 1993). The ability of *E. faecalis* to cause endocarditis was not affected by expression of aggregation substance or haemolysin.

However, mean vegetation weight was greater when animals were inoculated with cells expressing aggregation substance, whilst morbidity and mortality were significantly enhanced in animals infected with cells expressing haemolysin and aggregation substance in combination. This suggests that conjugative haemolysin plasmids of *E. faecalis* such as pAD1 are virulence factors in *E. faecalis* endocarditis (Chow *et al*, 1993).

In examining the complex antigenic composition of *E. faecalis* strains causing endocarditis, Aitchison *et al*, (1986) investigated two antigens, 37 and 43 kDa, which reacted strongly with an asparagus pea lectin-peroxidase conjugate on immunoblots, suggesting the presence of fucosyl residues. Antibodies to these antigens (later re-calculated as 37 and 40 kDa) along with a 73 kDa antigen were detected only in sera from patients with *E. faecalis* endocarditis, and not from other aetiological agents in infective endocarditis or in patients with *E. faecalis* infections other than endocarditis (Aitchison *et al*, 1987). Expression of the 37 and 40 kDa antigens appeared to be dependent upon growth medium, being expressed strongly in serum and to a lesser extent in brain heart infusion broth, but not in chemically-defined media. Furthermore, expression was demonstrated in vivo in a rabbit model (Lambert *et al*, 1990). A serodiagnostic ELISA based upon the partially purified 37, 40 and 73 kDa antigens successfully diagnosed 15 of 16 (94%) cases of *E. faecalis* endocarditis, and gave negative results in 9 of 10 cases of endocarditis due to other streptococci and 8 of 10 *E. faecalis* infections other than endocarditis (Shorrock *et al*, 1990). Thus the 37, 40 and 73 kDa antigens appear to be specific to *E. faecalis* endocarditis. Aside from being useful in diagnosis, the specificity of the antigens to endocarditis, and their expression in response to serum suggests a specific role in pathogenicity. Growth in serum mimics the environment the cells would encounter during bacteraemia, and may prime the cells for colonisation of the myocardium. Guzman *et al*, (1989; 1991a; 1991b) demonstrated that growth in serum increased adhesion to heart cells, and reduced interaction with polymorphonuclear leukocytes. These functions may be

associated with the serum-dependent changes in antigenic profile of *E. faecalis* noted by Lambert *et al*, (1990).

1.14. Aims and Objectives.

The aim of this project is to characterise the 37 and 40 kDa endocarditis specific antigens of *E. faecalis* identified by Aitchison *et al*, (1986). In doing so, it is hoped to gain further understanding of the pathogenic mechanisms of *E. faecalis* in infective endocarditis. *E. faecalis* endocarditis is increasingly difficult to treat, because of its ability to gain resistance to many of the currently available antimicrobial drugs. Identification of a function for the antigens may provide new therapeutic targets for the prevention or treatment of *E. faecalis* endocarditis.

Screening an *E. faecalis* genomic λZAP II library with antiserum from a patient with *E. faecalis* endocarditis (containing a high titre of antibodies against the 37 and 40 kDa antigens) will allow isolation of the structural gene(s) encoding the 37 and 40 kDa antigens. Cloning and nucleotide sequencing of the gene(s) will provide the inferred amino-acid sequence of the proteins, and computerised analysis of the these sequences may provide some insight into their structure and function.

The regulation of expression of the genes will be investigated by northern blot analysis of *E. faecalis* RNA, using a specific probe constructed from the cloned gene(s). Previous work has suggested that expression of the 37 and 40 kDa antigens is regulated by growth environment, in particular the presence of serum. Northern blot analysis will determine whether this effect is mediated at the transcriptional level. The nature of the inducing factor will also be investigated.

Chapter 2: Materials and Methods.

2.1. Bacterial strains

E. faecalis EBH1 was isolated from a patient (GP) with a severe case of enterococcal endocarditis at Birmingham Heartlands Hospital, Birmingham UK (Aitchison *et al*, 1986). Serum from this patient (GP serum), containing a high titre of antibodies against *E. faecalis*, was used to probe immunoblots and plaque lifts throughout. *E. faecalis* cells were grown in brain heart infusion (BHI, Difco) or 1% yeast extract (YE, Difco).

General cloning procedures were performed using the host strain *E. coli* XL1-Blue (*recA1, endA1, gyrA96, thi-1, hsdR17, supE44, relA1, lac,* [F', *proAB, lacI*^{q}, *ZΔ M15,* Tn*10*(tet^{r})]; Stratagene). Plaque screening was performed using *E. coli* SURE (*e14-(mcrA), Δ (mcrCB-hsdSMR-mrr)* 171, *sbcC, recB, recJ umuC::*Tn*5*(kan^{r}), *uvrC, supE44, lac, gyrA96, relA1, thi-1, endA1,* [F' *proAB, lacI*^{q}, *ZΔ M15*]; Stratagene). These strains are designed to allow use of blue/white selection for recombinants, and contain a *recA* mutation, which enhances the stability of cloned inserts. E. coli XL1-Blue also contains the F' episome, which contains genes for expression of F' pili, which are required for filamentous phage infection during single stranded DNA production. Bacterial growth media and other solutions are defined in appendix D.

2.2. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE).

Separation of *E. faecalis* proteins was carried out by electrophoresis in the Bio-Rad Protean and Mini Protean system, using denaturing 12% polyacrylamide gels (Lugtenberg *et al.,* 1975) prepared as shown in table 2.1.

Whole cell extracts were prepared by boiling cell suspensions with an equal volume of sample buffer for 10 minutes. Electrophoresis was carried out at 200V until the tracking dye, or appropriate prestained marker (see appendix C) reached the bottom of the gel.

Table 2.1. Composition Of Running Gel And Stacking Gel For SDS-PAGE.

Constituent	Running Gel (12%)	Stacking Gel	Sample buffer
Stock 1	5 ml	-	
Stock 2	-	2.5 ml	
SDS 10% W/v	0.5 ml	0.15 ml	5 ml
1.5M Tris pH 8.8	6 ml		
0.5M Tris pH 6.8		3.75 ml	2.5 ml
Dist, Water	8 ml	8 ml	5 ml
TEMED	50 µl	40 µl	
AMPS 10% W/v	70 µl	50 µl	
Glycerol			2.5 ml
2-mercaptoethanol			0.25 ml
5% Bromophenol blue		0.2 ml	

Stock 1: 44% W/v acrylamide and 0.8% W/v N,N'-methylene-bis-acrylamide (Bis)
Stock 2: 30% W/v acrylamide and 0.8% W/v N,N'-methylene-bis-acrylamide (Bis)
Final acrylamide concentration = 12% in the running gel and 3% in the stacking gel.

2.2.1. Visualisation of protein.

After electrophoresis, the gels were removed from the glass plates, and proteins either stained directly or transferred to nitrocellulose for subsequent probing with antisera (see Immunoblotting).

Directly stained gels were soaked with gentle agitation in 0.1% Coomassie Brilliant Blue R-250 (W/v) in 50% methanol/10% acetic acid for 30-60 mins. Gels were then destained in 10% methanol/20% acetic acid until background stain was removed. Gels could then be dried onto filter paper.

2.3. Immunoblotting.

Following separation by SDS-PAGE, cell components were transferred onto nitrocellulose membranes (0.45 μm pore size, Bio-Rad) according to the western blotting method of Towbin *et al* (1979). Transfer was carried out in a Mini Trans-Blot cell (Bio-Rad), in transblot buffer (25 mM Tris, 192 mM glycine and 20% $^{v}/_{v}$ methanol, pH 8.3), at 100V for one hour. Transfer efficiency was determined by Coomassie staining gels after transfer, or by staining nitrocellulose with naphthol blue black (0.7% $^{w}/_{v}$ in acetic acid).

Transferred proteins were immunodetected using serum from an endocarditis patient (GP) with a high IgG titre against *E. faecalis*. Blots were first washed for one hour with gentle agitation in TBS containing 1% bovine serum albumin (BSA). This blocked unbound sites on the nitrocellulose. Following this, blots were probed for at least three hours at 4°C with appropriate antisera diluted in TBS. Blots were then rinsed thoroughly in TBS three times, and soaked with gentle agitation in TBS/1% BSA containing 0.25 μg/ml protein-A conjugated either to horse radish peroxidase or alkaline phosphatase (Sigma) for 3 hours at 4°C. This solution was removed, and the blots rinsed again in TBS. Blots were visualised with freshly-prepared developing solutions. In the case of peroxidase conjugates, this was 0.01% H_2O_2 $^{v}/_{v}$ and 4-chloro-1-naphthol 25 μg/ml in 10 mM Tris. HCl pH 7.4). This solution could be warmed slightly to speed the reaction. Colour development was stopped with distilled water. For alkaline phosphatase conjugates, blots were first washed for 2 mins in colour development solution (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$), then incubated in the dark without shaking, in colour development solution containing NBT (0.3 mg/ml) and BCIP (0.15 mg/ml). Colour development usually took in excess of 30 minutes, and was stopped, to avoid high background, by washing with 20 mM Tris-HCl pH 2.9 containing 1 mM EDTA.

2.4. Isolation and purification of chromosomal DNA.

The method of Skjold *et al* (1987) was used. Cells from 50 ml overnight cultures of *E. faecalis* in Difco BHI were washed twice in 20 ml ice-cold 0.2M sodium acetate, centrifuged for 10 min at 2000 rpm and resuspended in 5 ml TE glucose. This cell suspension was incubated with mutanolysin (Sigma) 20 μg/ml for 2 hours at 37°C. This solution was centrifuged at 10,000 rpm for 15 minutes and the pellet gently resuspended in TE. N-Lauroylsarcosine (Sarkosyl) was added to 1% $^{w}/_{v}$ and the solution incubated at 37°C for 30 minutes. RNAase was added to 100 μg/ml and the incubation continued for 30 mins. Pronase (self digested) was added to 500 μg/ml and the incubation continued for a further 60 min. The resulting solution of cell contents was extracted repeatedly with phenol:chloroform (see appendix D) until no precipitate formed at the interface. The solution was then extracted once with chloroform:isoamyl alcohol (see appendix D.)

DNA was precipitated by addition of sodium acetate pH 5.2 to 0.3M, followed by two volumes of ethanol at -20°C. DNA was allowed to precipitate for 5 mins at room temperature and removed from solution by winding the thread-like precipitate onto a sterile pasteur pipette. The DNA was washed in 70% alcohol, dried under vacuum, and resuspended in TE.

2.5. Spectrophotometric determination of nucleic acid concentration.

For quantification of DNA or RNA, readings were taken at 260nm and 280nm. An OD of 1 at 260nm corresponds to a solution at 50 μg/ml for double stranded DNA, 40 μg/ml for single stranded DNA and RNA and ~20 μg/ml for single stranded oligonucleotides. Contaminating phenol and protein absorbs at 280nm. Nucleic acid solutions with a 260nm:280nm absorbance ratio of less than 1.8 were considered too impure for this method of quantification.

2.6. Digestion of DNA with restriction endonucleases.

DNA restrictions were performed using Pharmacia restriction enzymes and buffers, in accordance with manufacturer's instructions. For complete plasmid digestion, reaction mixtures were allowed to incubate at 37°C for 1 hour. Complete chromosomal digests were incubated for at least 4 hours. Partial digests are described in section 2.10.1.

2.7. Purification of DNA using Geneclean.

DNA was purified from agarose gels using the Geneclean system (Bio-101). Following agarose gel electrophoresis, the section(s) of the gel containing restriction fragment(s) of interest were excised. Four and a half volumes of 6M NaI and 0.5 vols of Geneclean-TBE modifier were added to the gel slice, and incubated at 50°C until the agarose melted. Five microlitres of Geneclean glassmilk suspension were added and the mixture incubated on ice for 5 minutes to allow DNA to adsorb onto the glass beads. Glassmilk beads were pelleted at 13,000 rpm for 5 seconds and washed three times with Geneclean 'NEW' wash. DNA was eluted from the beads into water or TE at 50°C for 5 minutes.

DNA in solution was purified by adding 3 volumes of 6M NaI and 5 µl of Geneclean glassmilk. After incubating on ice for 5 minutes, the glassmilk was pelleted at 13,000 rpm for 5 seconds, washed 3 times with Geneclean NEW wash, and the DNA was eluted into water or TE at 50°C for 5 minutes.

2.8. Ligation of DNA fragments into plasmid vectors.

The pBluescript phagemids are high copy number, ampicillin resistance plasmids. The polylinker is present in the N-terminal portion of a *LacZ* gene fragment,

such that strains containing plasmids with no inserts will grow as blue colonies on X-Gal agar, whereas plasmids with inserts give white colonies, due to disruption of the coding region of the *LacZ* gene fragment. pBluescript plasmids are available with two polylinker orientations, designated KS or SK. In the KS orientation, the restriction site closest to the *LacZ* promoter is Kpn I, and in the SK orientation, the first site is Sac I. Each version of the plasmid is also available as (+) or (-), according to which strand of the plasmid is produced as a single stranded copy, during infection by helper phage.

Plasmid vectors were linearised by restriction at a single site within the vector polylinker as described in section 2.6., and recovered from agarose gels using Geneclean as described in section 2.7. Fragments to be subcloned were similarly recovered to ensure purity. The DNA samples were mixed together, with a two to three fold molar excess of insert DNA over vector, and made up to 44 µl with water. To this was added 5 µl 10x ligase buffer, 0.5 µl 10 mM ATP and 0.5 µl (2 units) T4 DNA ligase. The mixture was incubated at 37°C for one hour in the case of sticky end ligations, or 12-20°C overnight for blunt end ligations.

2.9. Preparation of a horizontal agarose gel.

Agarose gels were cast and run in Bio-Rad DNA sub-cells. Ultrapure agarose (Bethesda Research Laboratories) was dissolved in 0.5x TBE buffer at 100°C. The solution was allowed to cool to approximately 60°C, and ethidium bromide was added to 0.5 µg/ml. The gel was poured and allowed to set at room temperature for 45 minutes. Once set, gels were submersed in 0.5x TBE. DNA samples were mixed with 0.2 vol 6x DNA loading buffer and electrophoresis was carried out at 70-200V until the tracking dye reached the end of the gel. Molecular weight markers were used where appropriate (see appendix C) DNA bands were visualised on a UV light box. Photographs were taken using Polaroid 655 film in a Polaroid MP4 camera.

2.10. Preparation of λZAP II library.

λZAP II is a cloning vector which will accept insert of up to 10 kb, and allows blue/white selection of recombinants. This system also simplifies subcloning, since cloned inserts can be rapidly converted into pBluescript phagemids.

To create a complete chromosomal library in λZAP II (linearised with *Eco*RI), it was necessary to digest partially the chromosome with *Alu*I (4 base recognition site), then ligate *Eco*RI linkers (Stratagene) to the *Alu*I digested blunt ended chromosomal DNA. Cutting the ligated linkers with *Eco*RI reveals the required sticky ends for ligation into λZAP II. Prior to cutting the linkers with *Eco*RI it was necessary to protect any *Eco*RI sites within the chromosomal digest by methylation.

2.10.1. Partial digestion of E. faecalis DNA with AluI.

To 60 µg of chromosomal DNA were added 12 µl of 10x reaction buffer (Stratagene) and 2 µl of *Alu*I (Stratagene 10 units/µl). Total volume was made up to 120 µl with sterile water and the reaction mixture mixed by pipetting and incubated at 37°C. 20 µl aliquots were removed at 10 minute intervals and the reactions stopped with 4 µl of DNA loading buffer. Samples were stored on ice until needed. After the final sample had been removed, all samples were run on a 0.8% agarose gel. Fragments between 2 kb and 10 kb were recovered.

2.10.2. Methylation of EcoRI sites within AluI digested DNA.

To 8 µg of recovered DNA was added 10 µl of 10x methylase buffer, 10 µl of 1M Tris buffer pH 8.0 and sterile water to 100 µl. Two 2 µl samples (labelled 1 and 2) were removed at this stage and stored on ice. To the bulk of the reaction was added 2 µl methylase (Stratagene 40 units/µl), then two more 2 µl samples, labelled 3 and 4, were removed. One hundred nanogrammes of pUC18 linearised with ScaI was added to all four control samples, then all five tubes were incubated at 37°C for 1 hour. Control samples were analysed as follows. To all four samples were added: 2 µl 0.1M

$MgCl_2$, 2 µl *Eco*RI reaction buffer and H_2O to 20 µl. To tubes 2 and 4 was added 1 µl of *Eco*RI 10 units/µl. All four tubes were incubated at 37°C for 1 hour, then analysed on a 1% agarose gel. If methylation and cutting were successful, lanes 1, 3 and 4 showed a single 2.69 kb band, whilst lane 2 showed bands at 1.72 and 0.96 kb.

2.10.3. Ligation of EcoRI linkers.

The *Eco*RI linkers used were double-stranded phosphorylated 12mers (Stratagene: dCCGGAATTCCGG). To 1 µg of blunt-ended methylated DNA were added 1 µl of linkers, 3 µl 10x ligase buffer, 3 µl 10 mM ATP pH 7.5, sterile water to 27 µl, and 3 µl DNA ligase (10 units/µl). The mixture was incubated at room temperature overnight. The DNA was then ethanol precipitated, resuspended in 10 µl of water and cut with 10 units *Eco*RI for 1 hour at 37°C. Excess linkers and cut fragments were removed by recovery from a 1% agarose gel.

2.10.4. Ligation into λZAP II arms.

To 1 µg of DNA were added 1 µl (1 µg) λZAP II arms, 0.5 µl 10x buffer, 0.5 µl 10 mM ATP, 0.5 µl T4 DNA ligase (4 units/µl) and sterile water to 5 µl. The mixture was incubated at room temperature overnight.

2.10.5. Packaging into λZAP II.

Packaging was according to manufacturer's instructions, using Gigapack II Gold packaging extracts (Stratagene). Following ligation, 4 µl of DNA was added to one melting 'freeze-thaw' extract. Fifteen microlitres of sonic extract was added and the tube contents mixed by pipetting. Packaging was allowed to continue for 2 hours at room temperature, then stopped with 500 µl of SM and 20 µl chloroform. The library was stored at 4°C. Phage were titred by plating out serial dilutions of the library (as described in section 2.11), and counting resulting plaques.

2.11. Immunoscreening of λZAP II plaques.

E. coli strain SURE was grown overnight (30°C shaking) in TB/10 mM $MgSO_4$/0.2% maltose). Cells were spun down at 2,000 rpm for 10 minutes, resuspended in 10 mM $MgSO_4$ to an OD_{600} of 0.5 and divided into aliquots of 200 µl. To each aliquot, phage suspension containing $15x10^3$ pfu was added, and incubated for 20 min at 37°C to allow phage to attach to cells. Each mixture was then added to 3.5 ml of molten top agar at 48°C and poured onto the surface of a pre-warmed and dried NZY plate. Plates were incubated for 3.5 hours at 42°C until plaques were just visible. Circular nitrocellulose filters (Stratagene), pre-soaked with 10 mM IPTG were placed onto the surface of the plates, which were allowed to incubate for several hours more at 37°C. At the end of this time, filters were marked (for orientation) and carefully removed. Plaque lifts were probed with GP antiserum diluted 1:500 in TBST/1% BSA at 4°C overnight. Immunoreactive clones were detected by incubating for three hours at room temperature with a protein A-alkaline phosphatase conjugate, then developing with BCIP and NBT as described in section 2.3. Colour development was complete within two hours. Reactive plaques were removed from plates with the wider end of a glass pasteur pipette and phage allowed to elute into 500 µl SM overnight. Phage suspensions were re-screened at low numbers to allow identification of discrete reactive plaques.

2.12. Verification of immunoreactive λZAP II clones.

Immunoreactive clones were characterised by affinity purification (Burnie and Clark, 1989). A lawn of *E. coli* SURE was infected with homogenous phage at numbers high enough to produce confluent lysis. Nitrocellulose filters were incubated with the lawn for 6 hours at 37°C, then washed, blocked and incubated with antiserum at 4°C overnight. After washing with TBS and then with 0.15M NaCl/0.05% Tween 20, antibodies were eluted with 5 ml of a solution of 0.15M NaCl, 0.05% Tween 20

and 0.2M glycine HCl pH 2.8 for 30 minutes then neutralised with 0.04g Tris. This was then diluted 1:1 in TBST/1% BSA and used to probe immunoblots of *E. faecalis* whole cell extracts as described in section 2.3.

2.13. In vivo excision of pBluescript from λZAP II.

The λZAP II genome includes the sequence of the pBluescript plasmid SK(-), into which inserts are cloned. This allows rapid recovery of cloned inserts as pBluescript phagemids as follows. Recombinant lambda are allowed to infect cells which are co-infected with helper phage R408 (a 4kb, single-stranded f1-type bacteriophage). Inside co-infected cells, bacteriophage derived proteins recognise initiator and terminator domains positioned within the lambda chromosome, flanking the pBluescript sequence, producing copies of pBluescript containing the cloned insert. These are released into the culture supernatant as single stranded phagemids, and can then be used to transform *E. coli* XL1-Blue.

Immunoreactive plaques were recovered as agar plugs from NZY plates and the phage eluted into 500 µl SM containing 20 µl chloroform overnight at 4°C. To 200 µl of the resulting phage suspension were added 200 µl of OD_{600} = 1 XL1-Blue cells and ~10^6 R408 helper phage. The mixture was incubated at 37°C for 15 minutes, then 70°C for a further 20 minutes. Cells were removed by centrifugation at 4000 rpm for 5 minutes, and the supernatant, containing packaged pBluescript, removed and stored at 4°C. To introduce the phagemids into XL1-Blue, 50 µl of a 10^{-2} dilution of the phage stock was mixed with 200 µl OD_{600} = 1 XL1-Blue at 37°C for 15 minutes, then plated onto LB agar containing ampicillin 100 µg/ml and tetracycline 12.5 µg/ml. Colonies contained double-stranded pBluescript harbouring the cloned insert.

2.14. Small scale preparation of bacterial plasmids (Minipreps).

Solution I = 50 mM glucose/ 25 mMTris/ 10 mM EDTA.
Solution II (aseptically prepared from stock solutions of 10N NaOH and 20% SDS) =0.2N NaOH/ 1% SDS. Both constituents were added to water to ensure that the SDS did not precipitate out of solution.
Solution III = 60 ml of 5M potassium acetate/ 11.5 ml glacial acetic acid/ 28.5 ml water.

One ml of an overnight bacterial suspension was centrifuged at 13,000 rpm for 1 minute. The supernatant was removed from each tube by using a pasteur pipette, 100 µl of solution I was added to each tube and the bacterial pellets resuspended by vigorous vortexing. Two hundred microlitres of freshly-prepared solution II were added, and the tube contents mixed by inverting gently five times. Tubes were placed on ice for 10 minutes, then 150 µl ice-cold solution III were added and mixed by gentle vortexing. The tubes were held on ice for 5 minutes and centrifuged at 13,000 rpm for 5 minutes. Supernatants were transferred to clean microfuge tubes, and extracted once with an equal volume of phenol:chloroform, and once with an equal volume of chloroform:isoamyl alcohol. The tube contents were mixed by vortexing, centrifuged at 13,000 rpm for 2 minutes and the aqueous upper layers were transferred to clean tubes. Plasmid DNA was precipitated by addition of 2 volumes of ethanol. The tubes were gently mixed, chilled for 5 minutes on ice, and then centrifuged at 13,000 rpm for 5 minutes. The supernatant was discarded, and the pellet was washed with 1 ml 70% ethanol. The supernatant was carefully decanted from each tube so as not to dislodge the pellet, and any residual ethanol was evaporated from the tubes under vacuum in a Speedvac SVC 100 set at medium drying temperature. Dried plasmid DNA was resuspended in 50 µl of water containing RNAase 20 µg/ml.

2.15. Preparation of electrocompetent *E. coli* XL-1 Blue cells.

The method of electroporation used was one outlined by Dower et al (1988). An overnight starter culture of *E. coli* XL-1 Blue cells was diluted 1:100 and grown to an OD_{600} of 0.5-1.0 in 500 ml LB medium. The cells were chilled briefly on ice and centrifuged at 4000 rpm for 15 min at 4°C. The ionic strength of the suspension was reduced by washing the cells in i) 500 ml ice cold sterile water, ii) 250 ml of sterile water, iii) 10 ml of sterile ice cold 10% glycerol, and finally resuspending in 0.5-1 ml of 10% glycerol. The ice-cold cell suspension was dispensed into 40 µl lots in sterile microfuge tubes and snap-frozen in liquid nitrogen. The electrocompetent cells were stored at -70°C until required.

2.16. Transformation of *E. coli* XL-1 Blue cells by electroporation.

The concentrated cells were thawed slowly on ice. Chilled recombinant plasmid DNA (up to 1 µg in water) was added, and the cell mix was pipetted into a sterile pre-chilled 0.2 cm electroporation cuvette (Bio-Rad Laboratories, Richmond, CA). The cuvette was placed in the pre-chilled cuvette carrier of a Gene Pulser and Pulse Controller (Bio-Rad) and subjected to a single 2.5 kV pulse, capacitance and resistance of the power supply having been set to 25 µ Farads and 200Ω, respectively, to produce a nominal time constant for the capacitor discharge of 5ms. Following electroporation, cells were allowed to recover in 1 ml SOC medium at 37°C with shaking for 1 hour (to allow expression of plasmid-coded antibiotic resistance). Transformed cells were selected by spreading onto L-agar plates containing ampicillin (100 µg/ml) and tetracycline (12.5 µg/ml), surface coated with 40 µl X-gal (20 mg/ml in DMF) and 4 µl 0.84M IPTG. After growing for 18 hours at 37°C, cells containing recircularised pBluescript plasmids appeared as blue colonies, recombinants grew as white colonies. From each transformation, several white colonies were picked with a sterile wire and grown overnight in LB broth containing ampicillin (100 µg/ml) and

tetracycline (12.5 μg/ml). Plasmid DNA was recovered (see section 2.14.) and analysed by restriction enzyme digestion.

2.17. Nucleotide sequencing.

Nucleotide sequencing was performed by the dideoxy mediated chain termination method of Sanger et al (1977). Sequencing reactions were performed using the Sequenase system (United States Biochemical), using either single-stranded or double-stranded template DNA. Subsequent electrophoresis was performed using the Bio-Rad Sequi-Gen system (21cm x 40cm x 0.04cm).

2.17.1. Preparation of single-stranded template DNA.

Single-stranded DNA was generated using the Stratagene pBluescript phagemid system according to manufacturer's instructions. Briefly, cells with the pBluescript phagemid containing DNA to be sequenced were grown to early log phase (OD_{600} = 0.1) and infected with helper phage R408 (multiplicity of infection ≥ 1:1). After at least 8 hours growth, cells were removed by centrifugation, and the culture supernatant removed and stored at 4°C.

To each 1.2 ml of supernatant was added 300 μl of 3.5M ammonium acetate, pH 7.5, 20% polyethylene glycol 8000 (Sigma). The tube contents were mixed by inversion and phage were allowed to precipitate at room temperature for 20 min. Following by centrifugation at 13,000 rpm for 20 min, the phage pellet was resuspended in 300 μl TE, and subjected to repeated extractions with phenol:chloroform until no more insoluble material was produced at the interface. Following one further extraction with chloroform:isoamyl alcohol, ssDNA was precipitated by addition of an equal volume of 7.5M ammonium acetate, pH 7.5 and 2 volumes cold 100% ethanol, and allowed to stand at -70°C for 30 min. ssDNA was pelleted at 13,000 rpm for 30 minutes, washed in 70% ethanol, dried under vacuum,

and resuspended in 20 µl water. The ssDNA was analysed on a 1% agarose gel; R408 runs at approx. 4 kb, while pBluescript with no insert runs at approximately 1.6 kb when compared to a double-stranded DNA marker.

2.17.2. Preparation of double-stranded template DNA.

Double-stranded plasmid DNA was denatured in 0.2M NaOH, 0.2 mM EDTA at 37°C for 30 minutes, then precipitated with 0.1 vols 3M sodium acetate pH 5.2 and 2-4 volumes of ethanol. DNA was microfuged at 13,000 rpm for 30 minutes, washed with 70% ethanol and dried under vacuum before resuspending in 7 µl sterile water.

2.17.3. Sequencing reactions.

Template DNA was sequenced using the Sequenase (version 2.0) kit according to the manufacturer's instructions. For single-stranded templates, approximately 1 µg of DNA was used, for double-stranded templates, 10-20 µg was used. Primers were 17-mers, complimentary to pBluescript sequences flanking the polylinker. Half a picomole of primer was used per reaction.

2.17.4. Denaturing gel electrophoresis.

Polyacrylamide gel electrophoresis was performed with the Bio-Rad Sequi-Gen nucleic acid sequencing cell. Samples were first denatured at 75-80°C for three minutes, then resolved on a 7M urea, 6% polyacrylamide gel.

The Sequi-Gen apparatus was thoroughly cleaned to remove all traces of grease, and one plate was siliconised with 500 µl of Sigma Cote to prevent gels from sticking to both plates. Cells were assembled with 0.4 mm spacers, and the bottom sealed with a plug gel consisting of 10 ml 6% acrylamide solution (Sequagel-6 National Diagnostics) polymerised with 50 µl of 25% $^{W}/_{v}$ AMPS and 50 µl TEMED. Forty millilitres of acrylamide solution catalysed with 40 µl of 25% AMPS and 40 µl TEMED were poured between the plates. A sharktooth comb (Bio-Rad) was inserted,

flat side first, 5mm into the gel. Once polymerisation was complete, the comb was removed, the top of the gel was washed with 1x TBE.

Gels were pre-run at 40-50W for at least one hour, then 2-3 µl of each sample loaded. Electrophoresis was at constant power (40-50W) to maintain gel temperature at 50°C throughout the run. Once run, the apparatus was disassembled and plates prised apart. Gels were immersed in 5% acetic acid/15% methanol for 30 minutes (to remove urea), then transferred to 3mm Whatman paper, covered in cling film and dried under vacuum at 80°C for 45 minutes.

2.17.5. Autoradiography.

After drying, gels were exposed to Kodak X-omat AR film for 24-48 hours at room temperature in a light-proof cassette. After exposure, film was developed according to the manufacturer's protocol.

2.18. Analysis of Nucleotide sequence.

The deduced nucleotide sequence of *efaA* was submitted to the GenBank using the Authorin package. Subsequent DNA sequence analysis was performed using the GCG (University of Wisconsin Genetics Computer Group) package at the SERC Seqnet computing facility at Daresbury, UK. Genbank database searching was performed using the FASTA and TFASTA software, multiple sequence alignments were assembled using the CLUSTALV and ALIGN programs. Additional sequence analyses were performed using the PCGENE package (Intelligenetics).

2.19. Preparation of ^{32}P-labelled DNA probe for hybridisation reactions.

A 405 bp *Hin*dIII fragment of *efaA* was generated by digestion of plasmid pGP19 with *Hin*dIII, followed by recovery of the fragment from an agarose gel using Geneclean. This DNA was used as a template for generation of an [α-^{32}P]dATP labelled probe using DNA polymerase primed by random hexanucleotides. Approximately 200 ng of purified DNA in 9 µl water were added to 2 µl random hexanucleotide mixture (Boehringer), 3 µl of a mixture of dTTP, dCTP and dGTP, all at 20 mM (Pharmacia), 5 µl [α-^{32}P]dATP (3000 Ci/mmol, 10 µCi/µl) and 1 µl (10 units) Klenow fragment of DNA polymerase. The reaction mixture was incubated at 37°C for 30 minutes and stopped by heating to 65°C for 5 minutes. Unincorporated nucleotides were removed by passing the final mixture through a Nuc-Trap column (Stratagene). This method generates probes of very high specific activity 10^8-10^9 cpm/µg. Such high activity probes were used within a week of synthesis, as high energy α-^{32}P emissions can cause destruction of the molecular structure of the nucleotide sequence.

2.20. Southern blotting.

Digested DNA was transferred from agarose gels to nitrocellulose as described by Southern (1975). Briefly, the DNA was depurinated in 0.25M HCl for 30 minutes and denatured with 0.6M NaCl, 0.2M NaOH for 30 minutes. Neutral pH was restored with 1.5M NaCl, 0.5M Tris-HCl (pH 7.0) for 30 minutes. Fragments were then blotted by capillary transfer of 20x SSC overnight (Southern, 1975). DNA was fixed onto the membranes by baking at 80°C for 2 hours *in vacuo*. Filters were wetted in 5x SSC, rolled in nylon mesh and inserted into Hybaid hybridisation bottles. Blots were pre-hybridised rotating in a Hybaid mini-hybridisation oven at 50°C in hybridisation solution (1% blocking reagent (Boehringer Mannheim), 5x SSC, 0.1% ^{w}/v Sarkosyl, 0.02% ^{w}/v SDS) for at least one hour. This was then removed, and replaced with 10

ml hybridisation solution containing 140 µl radiolabelled probe, prepared as described in section 2.19., denatured by heating to 95°C for 5 minutes. Following overnight hybridisation, filters were washed twice for 5 minutes at room temperature with 2x SSC containing 0.1% SDS, then twice for 10 minutes each with 0.1x SSC containing 0.1% SDS at 55°C (low stringency), 60°C (medium stringency) or 68°C (high stringency). Following stringency washes, blots were wrapped in cling film, and exposed to Kodak X-omat AR film at -70°C with intensifying screens for 6-48 hours, then developed according to manufacturer's instructions.

2.21. RNA analysis of *E. faecalis* EBH1.

In order to study regulation of expression of *efaA* at the transcriptional level, total cellular RNA was extracted from EBH1 following growth in a range of conditions, separated in denaturing agarose gels, transferred to nitrocellulose (Northern blotting) and probed with a ^{32}P-labelled 405 bp internal restriction fragment of *efaA*. Prokaryotic mRNA is highly susceptible to degradation by RNAases, which are ubiquitous, and not destroyed by autoclaving alone. Hence all glassware used in RNA procedures was baked at 180°C for 8 hours prior to use. All solutions were treated with 0.1% diethyl pyrocarbonate (DEPC, a potent inhibitor of RNAases) overnight at 37°C, then autoclaved. All other equipment (including electrophoresis tanks, blotting tanks and hybridisation tubes) was soaked in 0.1% DEPC overnight at 37°C.

2.21.1. Preparation of total cellular RNA.

Total RNA was prepared by the hot phenol method (Tanimoto and Clewell, 1993). An overnight culture of *E. faecalis* EBH1 in 1% yeast extract (Difco) was diluted 1:100 into pre-warmed medium and grown to mid log phase (OD_{600}= 0.5-1). Cells were chilled on ice, harvested and resuspended in 600 µl lysis buffer (20 mM

Tris-HCl (pH 8.0), 3 mM EDTA, 200 mM NaCl).. To this was added 60 µl 10% SDS, and the mixture was maintained at 100°C for two minutes, with mixing. Six hundred microlitres of hot phenol saturated with lysis buffer were added, and the mixture maintained at 65°C for 5 minutes, with occasional mixing. Phases were separated by centrifugation, and the aqueous phase extracted twice further with phenol chloroform. RNA was precipitated with 0.1 volume of 3M sodium acetate and two volumes of ethanol, and resuspended in DEPC-treated water.

2.21.2. Denaturing agarose electrophoresis.

In order to maintain denaturing conditions during electrophoresis of RNA samples, formaldehyde/agarose gels were used. All procedures involving formaldehyde and formamide were carried out in a chemical hood. To 30 ml of a molten 1.28% agarose solution (DEPC treated) was added 9.4 ml of 5x formaldehyde gel-running buffer (0.1M MOPS (pH 7.0), 40 mM sodium acetate, 5 mM EDTA pH 8.0) and 8.6 ml of 12.3M formaldehyde. Gels were cast, and allowed to set for at least 30 minutes at room temperature.

RNA samples for electrophoresis were prepared as follows. Five to ten micrograms of RNA in 4.5 µl water were added to 2 µl 5x formaldehyde running buffer, 3.5 µl formaldehyde and 10 µl formamide in a sterile microfuge tube and held at 65°C for 15 minutes then chilled on ice. Two microlitres of formaldehyde gel-loading buffer (50% glycerol, 1 mM EDTA, 0.25% bromophenol blue), and 1 µl of ethidium bromide 1 mg/ml were added, and the samples electrophoresed at 10-20 V/cm. Gels were viewed on UV light and photographed if required.

2.21.3. Northern blotting.

Transfer of RNA from denaturing agarose gels to nitrocellulose filters was carried out by a method similar to that of Southern blotting. All apparatus was treated with DEPC prior to use. Gels were soaked for 20 minutes in 0.05N NaOH, then rinsed

in DEPC-water and soaked in DEPC 20x SSC for 45 minutes. Gels were then placed in contact with nitrocellulose and RNA transferred by capillary flow of 20x SSC as described for Southern blotting. Following transfer, nitrocellulose filters were oriented, washed in 5x SSC, then baked for 1 hour at 80°C *in vacuo*.

2.21.4. Northern hybridisation.

After baking, filters with bound RNA were incubated for at least 1 hour at 45°C with northern pre-hybridisation buffer (7% SDS, 50 mM sodium phosphate buffer pH 7.0, 2% blocking reagent (Boehringer Mannheim) 5x SSC, 0.1% Sarkosyl). Filters were then hybridised with heat-denatured ^{32}P-labelled probe in 10 ml northern pre hybridisation buffer at 45°C overnight. Probe solutions were removed and filters were twice washed for 5 minutes at room temperature in 2x SSC containing 0.1% SDS, then twice further for 15 minutes at 65°C in 0.1x SSC containing 0.1% SDS. Filters were developed as described for Southern blotting. Following autoradiography, blots were stained with methylene blue to verify loadings. Dried filters were first soaked in 5% acetic acid for 10 minutes, then transferred to 0.04% ^{w}/v methylene blue in 0.5M sodium acetate (pH 5.2) for 10 minutes at room temperature and washed with distilled water.

2.22. Expression from cloned DNA.

Overnight cultures of *E. coli* GP19- and *E. coli*/pSK- were diluted 1:100 in pre-warmed LB medium and grown at 37°C with shaking for 2.5 hours. Pre-induction samples were removed (1.5 ml) and stored on ice. IPTG was added to 10 mM and growth allowed to continue. Further samples were taken 1, 2, 3 and 4 hours post induction and stored on ice until needed. Cells were harvested, resuspended at equal concentration in saline and whole cell extracts were subjected to SDS-PAGE and immunoblot analysis as described in section 2.2.

2.23. Preparation of dialysis tubing

Prior to use, dialysis tubing was cut to an appropriate length and boiled for 10 minutes in 2% $^{W}/_{v}$ $NaHCO_3$ then placed into capped 20 ml universal tubes submersed in double distilled water and autoclaved for 15 minutes at 121°C.

2.24. Scanning densitometry

Autoradiographs and stained blots were scanned using a Bio-Rad scanning densitometer. Analysis of the digitised images was performed using the Bio-Rad Molecular Analyst software package. Molecular weights were determined using Profile Analysis software (Bio-Rad) in comparison with molecular weight standards. Quantification of bands was determined using Volume Analysis software (Bio-Rad).

2.25. Bacterial adherence to hydrocarbon (BATH) assay.

Bacterial hydrophobicity was assessed by a modification of the bacterial adherence to hydrocarbon (BATH) assay, as described by Rosenberg *et al*, (1980). Cells were harvested, washed twice, and resuspended to OD_{600} = 0.6 in 10 mM NaH_2PO_4/NaOH buffer (pH 7.0). Six hundred microlitres of cell suspension was added to an equal volume of hexadecane (BDH), and vortex mixed for 2 minutes. After phase separation (30 minutes standing at room temperature), the decrease in OD_{450} of the aqueous phase was used as a measure of cell surface hydrophobicity.

Chapter 3: Cloning and expression.

The first step in characterising the 37 and 40 kDa endocarditis-specific antigens of *E. faecalis* was to isolate clones containing their structural gene(s). Since the only available means of identifying the antigens was polyclonal antiserum from an endocarditis patient, this was used to screen random λZAP II clones created from *E. faecalis* EBH1 genomic DNA. The work detailed below describes the isolation of recombinant lambda phage reactive with the patient antiserum, selection of the appropriate clones, and expression of protein from these clones in an *E. coli* host.

3.1. Construction of an EBH1 genomic library in λZAP II.

Chromosomal DNA was extracted from *E. faecalis* EBH1 as described in section 2.4. In order to ensure a fully representative library it was necessary to use a partial *Alu*I chromosomal digest and ligate into *Eco*RI-cut λZAP II with the aid of synthetic linkers. Analysis of the constructed phage library on IPTG/X-Gal plates showed that it contained 7.5×10^6 phage, (15,000 pfu/μl) of which 15% (1.13×10^6) were recombinants. The probability of creating a recombinant containing the sequences of the gene of interest in the correct frame and orientation for expression is:

$$P = \frac{\text{size of the coding region of the gene of interest}}{\text{size of genome (kb) x 6}}$$

Where P is the probability of creating a recombinant containing the sequence of interest in the correct reading frame and orientation for expression (Sambrook *et al*, 1989). For a 40 kDa protein, the DNA coding region would be approximately 1.2 kb

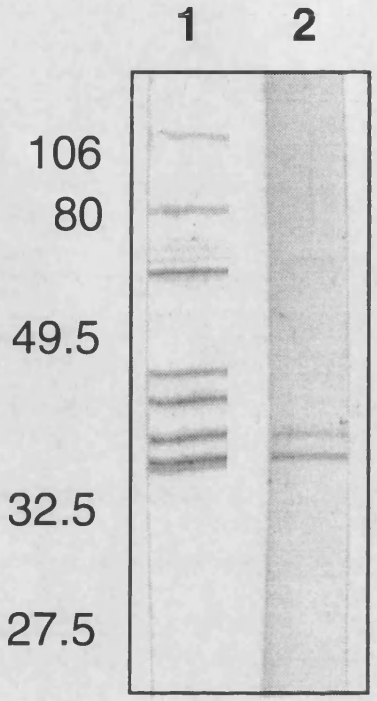

Figure. 3.1. Immunoblot of *E. faecalis* EBH1 whole cells probed with serum from patient GP with *E. faecalis* endocarditis (lane 1), and antibodies affinity purified from immunoreactive plaque (lane 2).

long. The genome size of *E. faecalis* is approximately 2,500 kb (Miranda *et al* 1992). Thus in this case $P = 8x10^{-5}$. The minimum number of recombinants to be screened is given by 1/P, ie 12,500.

3.2. Immunoscreening of λZAP II plaques.

Phage were plated at 15,000 pfu per 82mm plate, lifted onto nitrocellulose circles and screened with *E. coli* lysate-adsorbed GP antiserum (diluted 1:500 in TBS/1% BSA) according to the *pico*Blue screening protocol (Stratagene). Screening 20 plates (45,000 recombinants) revealed 29 immunoreactive plaques which were removed, eluted into SM buffer and purified by further screening at low numbers.

3.3. Verification of λZAP II immunoreactive clones.

GP antiserum contained a high titre of IgG to a number of *E. faecalis* EBH1 proteins, including the 37, 40 and 73 kDa proteins discussed earlier. This presented the problem of not knowing which, if any, of the immunoreactive clones contained genes encoding the proteins of interest. This was resolved using the affinity purification protocol described by Burnie and Clark (1989). Immunoreactive phage were plated at 15,000 pfu per plate and lifted onto nitrocellulose circles according to the immunoscreening protocol. After incubation with GP antiserum, antibodies were eluted, and used to probe strip blots of whole cell extracts of *E. faecalis* EBH1. One clone out of the 29 initial positives showed reactivity with both the 37 and 40 kDa proteins (figure 3.1.). This clone, designated GP19 was rescued in vivo as a pBluescript SK- phagemid and designated pGP19-. The corresponding SK+ phagemid was designated pGP19+. Phagemids were transformed into *E. coli* XL1-Blue.

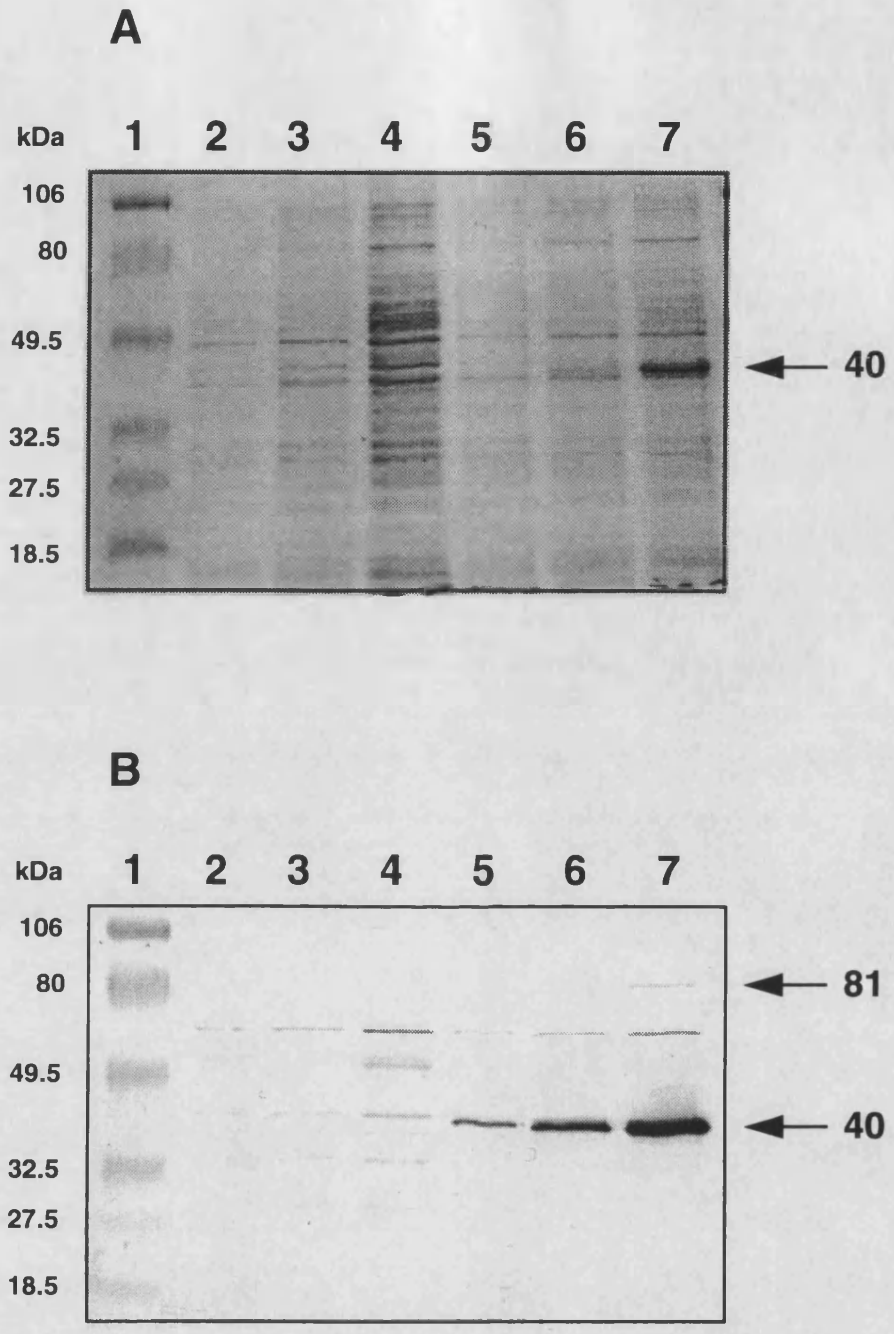

Figure 3.2 SDS-PAGE (panel A) and immunoblot (panel B) analysis of whole cell lysates from *E. coli* XL1-Blue/pSK- vector (lanes 2-4) and *E. coli* XL1-Blue/pGP19 (lanes 5-7) before and after induction with 10mM IPTG. Lane 1, molecular weight markers; lanes 2 and 5, pre induction; lanes 3 and 6, 1 hour induction; lanes 4 and 7, 4 hours induction.

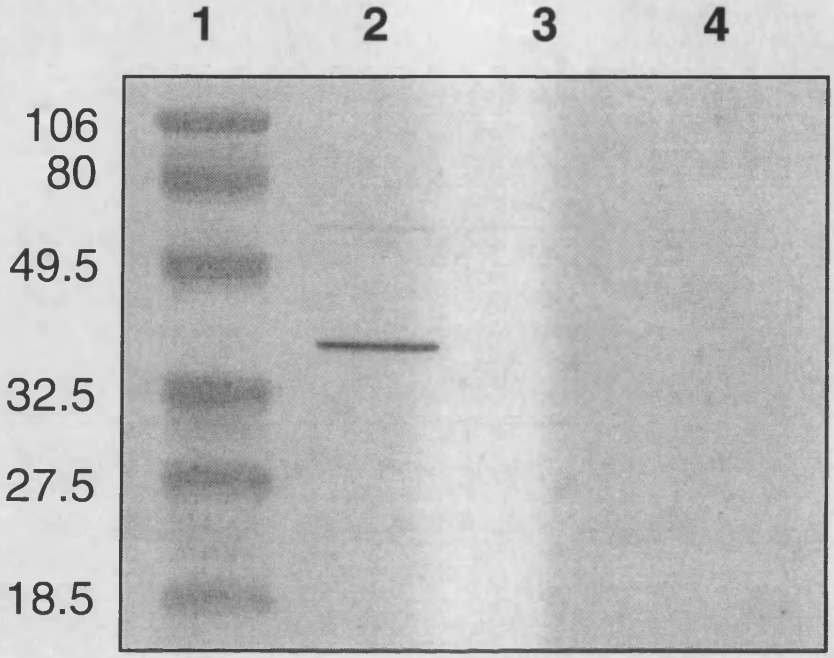

Figure 3.3. Immunoblot analysis of whole cell lysates from *E. coli* XL1-Blue/pGP19 (lane 2), XL1-Blue/pGPH2 (lane 3) and XL1-Blue/pSK- vector (lane 4), after 1h induction with 10 mM IPTG, probed with serum from patient GP with *E. faecalis* endocarditis. Lane 1 contans molecular weight markers.

3.4. Restriction analysis of pGP19+/-.

Digestion of plasmid pGP19 with *Eco*RI revealed a single 1.7 kb insert in the pBluescript polylinker. pGP19 was then digested with all other available enzymes (*Kpn*I, *Bam*HI, *Xho*I, *Xba*I, *Eco*RV, *Sma*I, *Hin*dIII, *Sal*I, *Sca*I, and *Pst*I) of which only *Hin*dIII cut within the insert, at 365 bp and 770 bp.

3.5. Expression from cloned DNA.

The cloned inserts in the pGP19 plasmids are under the control of the *lacZ* promoter, which suppresses expression in the absence of inducer, isopropylthio-β-D-galactopyranoside (IPTG). To examine the products of any open reading frames in the cloned inserts, expression was induced with 10mM IPTG as described in section 2.22. Whole cell extracts of induced *E. coli* XL1-Blue/pGP19 cells were analysed by SDS-PAGE and western blotting, and compared with the vector pSK- as a control. SDS-PAGE revealed the presence of an approximately 40 kDa protein, which reacted strongly with GP antiserum on western blots (figure 3.2.). The protein was expressed weakly in the absence of IPTG, but maximal expression was achieved after 4h induction. The vector control was negative.

In order to determine which region of pGP19 was responsible for expression of the immunoreactive product, the 1.7 kb insert was restricted with *Hin*dIII and *Eco*RI, and the smaller fragments unidirectionally subcloned as described in section 2.8. Correct orientation was established by nucleotide sequencing of the subclones. Expression was detected from pGP19, but not from plasmid pGPH2 (which contains an approximately 1kb fragment from the 3' end of the pGP19 insert)(figure 3.3.). Thus it was deduced that the start codon of the open reading frame encoding the immunoreactive protein was within the first 700 bp at the 5' end of the insert.

3.6. Discussion.

3.6.1. Isolation of an immunoreactive clone.

Screening a genomic library of *E. faecalis* EBH1 with serum from an *E. faecalis* endocarditis patient produced several immunoreactive clones. Affinity purification demonstrated that one of these clones was cross reactive with both the 37 and 40 kDa antigens of *E. faecalis* which have been shown to be of use in the serodiagnosis of infective endocarditis (Shorrock *et al* 1990, Aitchison *et al* 1987). This clone, designated GP19, was selected for subcloning and further analysis. Cross reactivity with the two antigens may have been due to GP19 containing the structural genes for both peptides, indicating that the 37 and 40 kDa proteins are from the same, or very close operons. This however, is not the case, since a single immunoreactive approximately 40 kDa protein is expressed from pGP19-, suggesting rather that the two *E. faecalis* antigens are antigenically very closely related. It is possible that the two antigens represent different forms of the same peptide, perhaps due to cleavage of a leader peptide. Conversely, the heavier protein may represent post-translational modification of the parent peptide, possibly by glycosylation. Previous studies on the 37 and 40 kDa antigens have suggested some glycosylation of antigenic epitopes (Aitchison *et al*, 1986; 1987). Attempts were made to obtain the N-terminal amino acid sequences of the 37 and 40 kDa antigens by automated peptide microsequencing (Edman degradation), for comparison with the inferred amino acid sequence of the cloned gene so that this hypothesis could be tested. However, these attempts were unsuccessful, so no comparison could be made.

Bacterial proteins are frequently expressed as precursors which are modified substantially before achieving their final active conformation and location. This post-translational modification often involves a leader sequence which directs the immature peptide to a particular cell location, before being cleaved from the mature protein by a specific signal peptidase enzyme (Dawes and Sutherland, 1992). Removal of the

leader may also involve addition of other components. This raises the possibility that the 37 kDa antigen may represent the 40 kDa antigen with the leader sequence removed.

As well as the 40 kDa band, an immunoreactive 81 kDa band became visible after 4 hours induction of pGP19 (figure 3.2.). The band was not present in vector control cells. This may be due to aggregation of the cloned gene product into dimers, as has been shown for other similarly sized streptococcal surface proteins (Ganeshkumar *et al*, 1988; Oligino and Fives-Taylor, 1993). These polymeric forms were shown to be resistant to boiling in SDS and mercaptoethanol, as was the case for the pGP19 product. The nature and significance of the polymeric forms is not understood, but in the case of FimA, a fimbrial adhesin of *S. parasanguis*, it may be concerned with self assembly of fimbrial structural subunits (Oligino and Fives-Taylor, 1993). Polymerisation into larger units may also allow these relatively small proteins to be exposed at the surface of the cell, despite the presence of a thick cell wall (Jenkinson, 1994).

3.6.2. Expression from pGP19 in the absence of IPTG.

pUC-related cloning vectors, such as pBluescript, contain a polylinker of unique restriction sites oriented such that transcription of cloned DNA is under the influence of the *lacZ* promoter. In the absence of inducer, transcription is repressed to prevent the build up of potentially toxic products in the cell, whilst in the presence of inducer, such as IPTG, transcription occurs rapidly, allowing expression of large quantities of protein.

The addition of 10mM IPTG to a log phase culture of *E. coli* XL1-Blue/pGP19 caused a large increase in production of the gene product, however some low level expression also occurred in the absence of IPTG. These data suggest that expression from pGP19 is under the influence of its own promoter, requiring *E. coli* RNA polymerase to recognise streptococcal promoter elements. The ability of *E. coli*

transcription and translation mechanisms to accept more divergent promoter and initiation sequences than other (particularly Gram-positive) species is well recognised (McLaughlin *et al*, 1981; Moran *et al*, 1982). The expression seen in the absence of IPTG is weak, as would be expected if consensus sequences differ between species. A more likely explanation is that areas of high A-T content within the insert act as weak promoters. This situation is well documented among species of high chromosomal A-T content such as *S. pneumoniae* (Morrison and Jaurin 1990; Dillard and Yother 1991). Random segments of *S. pneumoniae* DNA cloned into promoter probe plasmids in *E. coli* exhibit a higher frequency of strong promoter activity than similarly cloned *E. coli* fragments (Chen and Morrison 1987). This is explained by a higher frequency of random sequences falling into the *E. coli* consensus promoter sequences (TTGACA and TATAAT) within the A-T rich *S. pneumoniae* chromosome. If this hypothesis is correct, then the *S. pneumoniae* RNA polymerase must have a more stringent consensus sequence requirement than that of *E. coli*. Indeed, Dillard and Yother (1991) have noted that *S. pneumoniae* sequences which act as promoters in *E. coli* do not necessarily do so in *S. pneumoniae*, whereas actual promoter sequences of *S. pneumoniae* may have increased strength in *E. coli* (McLaughlin *et al* 1981; Moran *et al* 1982). Enterococcal DNA has a similarly high A-T content (58-66%, Holt *et al*, 1994), therefore it may be assumed that similar weak promoter sites exist in cloned *E. faecalis* DNA at a similar frequency to that seen in *S. pneumoniae*, with such sites resulting in low level expression of cloned inserts in *E. coli*, as is seen in this case. Although speculative, these findings suggest that stronger expression might have been expected had a true promoter been cloned.

A further manifestation of false promoter elements in cloned A-T rich DNA is that excessive transcription can interfere with plasmid replication and expression of plasmid-encoded genes. As a result, plasmids become unstable, and cloning of certain sequences becomes difficult unless transcriptional terminators are included (Dillard and Yother, 1991). Such difficulties were encountered during subcloning of pGPH1-

for nucleotide sequencing. The problem was solved by using pBluescript KS-/+, a version of the plasmid in which the polylinker sequence is reversed.

3.6.3. Serodiagnosis of E. faecalis endocarditis.

Diagnosis of the causative agent in endocarditis is an essential step in ensuring that the patient receives the appropriate antibiotic therapy. Blood cultures usually give the most accurate diagnosis, however, these can take up to 24 hours, and may be negative in certain cases (Tunkel and Kaye, 1992). Any technique capable of rapidly identifying or eliminating specific organisms in infected patients would be of great clinical value. The use of immunological methods in diagnosis provides a sensitive, specific and rapid alternative to culture techniques, especially in specimens where organisms cannot easily be identified by culture, such as sputum, or in cases where aggressive antibiotic therapy has been initiated (Lambert, 1990). Shorrock *et al*, (1990) devised an ELISA based upon the 37, 40 and 73 kDa endocarditis specific antigens of *E. faecalis* which successfully diagnosed *E. faecalis* endocarditis, whilst giving negative results for patients with either endocarditis due to other streptococci or *E. faecalis* infections other than endocarditis.

Since the cloned *E. faecalis* protein was shown to be cross reactive with the 37 and 40 kDa antigens, then it may be of use in the serodiagnosis of endocarditis. The use of cloned protein in an ELISA would offer certain advantages over the use of purified whole cell extracts. Shorrock *et al*, (1990) partially purified the 37, 40 and 73 kDa antigens from crude whole cell extracts of *E. faecalis* by ammonium sulphate precipitation. In a recombinant system, much larger quantities of a single, pure protein can be produced, which should give more reliable, reproducible results than cell extracts, where accurate reproduction of cell growth conditions and extraction procedures would be necessary. In the absence of N-terminal amino acid sequence for the 37 and 40 kDa antigens, the use of the cloned gene product in serodiagnosis may

give further indication of the relationship with the 37 and 40 kDa antigens of *E. faecalis*.

In summary, a gene expressing an approximately 40 kDa protein has been cloned, which reacts strongly with antiserum from an *E. faecalis* endocarditis patient, and is cross reactive with both the 37 and 40 kDa antigens of *E. faecalis*. The nature of this protein will be determined by sequence analysis of the gene(s) within the cloned DNA insert.

Chapter 4: Sequence analysis.

In this chapter, the determination of the nucleotide sequence of pGP19, and its subsequent analysis by translation into an amino acid sequence, and comparison with sequence databases is described. It is hoped that the data presented here, and in the subsequent discussion will provide an explanation of the results described in chapter 3, and may also suggest a role for the cloned protein in the pathogenesis of *E. faecalis*.

4.1. Nucleotide sequencing of pGP19+/-.

Nucleotide sequencing was performed using single-stranded templates generated from the pBluescript phagemid system. Since plasmid pGP19- was too large to sequence directly without the aid of synthetic primers, it was digested, and subcloned in smaller pieces. Plasmid pGP19- consists of 3 *Hin*dIII fragments, each of which was subcloned unidirectionally into pSK- and pSK+, giving plasmids pGPH1+/-, pGPH2+/- and pGPH3+/-. Plasmids pGPH1+/- and pGPH3+/- contain 369 bp and 405 bp, respectively, and could be sequenced from commercially obtained pSK primers. pGPH2+/- required the manufacture of synthetic primers to allow sequencing. The partial nucleotide sequence of pGP19 is shown in figure 4.1.

```
        10         20         30         40         50         60
GAATTCCGGCCGGAATTCCGGCTTCTGGTGCGACGATTGTTTTAACCGCCGCCTTATTCT      60

TTTTATTGGCTTTCTTTTTCTCACCAAAGAAAGGCCTAGTATTTGTAAACCGTGAGAAAG     120

AAATGGAGGAATCAACGAATGAAAAAATTTAGTTTATTTTTTTTAACACTTTTAGCAGGG     180
      SD          M  K  K  F  S  L  F  F  L  T  L  L  A  G

TTAACGTTAGCTGCTTGCGGGAATCAAGCCGCTGAAAAGAAAGAAAAATTAGCAATTGTG     240
L  T  L  A  A  C  G  N  Q  A  A  E  K  K  E  K  L  A  I  V

ACAACGAACTCGATCTTATCCGATTTAGTGAAAAATGTTGGGCAAGACAAAATTGAGCTG     300
T  T  N  S  I  L  S  D  L  V  K  N  V  G  Q  D  K  I  E  L

CATAGTATTGTGCCAATTGGGACAGACCCTCACGAATATGAACCGTTACCAGAAGACATT     360
H  S  I  V  P  I  G  T  D  P  H  E  Y  E  P  L  P  E  D  I

GCGAAAGCTTCTGAAGCGGACATTTTATTCTTTAACGGCTTGAACTTAGAAACAGGCGGA     420
A  K  A  S  E  A  D  I  L  F  F  N  G  L  N  L  E  T  G  G

AATGGCTGGTTTAACAAATTAATGAAAACGGCCAAAAAAGTTGAGAATAAAGATTACTTT     480
N  G  W  F  N  K  L  M  K  T  A  K  K  V  E  N  K  D  Y  F

TCTACAAGCAAAAATGTTACGCCACAATATTTAACAAGTGCCGGTCAAGAACAAACAGAA     540
S  T  S  K  N  V  T  P  Q  Y  L  T  S  A  G  Q  E  Q  T  E

GATCCGCATGCTTGGTTAGACATTGAAAATGGCATCAAATATGTAGAAAACATTCGTGAC     600
D  P  H  A  W  L  D  I  E  N  G  I  K  Y  V  E  N  I  R  D

GTGTTAGTAGAAAAAGATCCAAAAAATAAAGATTTCTATACAGAAAACGCGAAAAATTAT     660
V  L  V  E  K  D  P  K  N  K  D  F  Y  T  E  N  A  K  N  Y

ACCGAAAAACTTAGCAAACTACATGAGGAAGCCAAAGCTAAATTTGCTGATATTCCTGAT     720
T  E  K  L  S  K  L  H  E  E  A  K  A  K  F  A  D  I  P  D

GATAAAAAATTATTAGTTACAAGTGAAGGTGCCTTTAAATATTTCTCCAAAGCTTATGAT     780
D  K  K  L  L  V  T  S  E  G  A  F  K  Y  F  S  K  A  Y  D

TTAAATGCCGCTTATATTTGGGAAATTAACACAGAAAGTCAAGGAACACCTGAACAAATG     840
L  N  A  A  Y  I  W  E  I  N  T  E  S  Q  G  T  P  E  Q  M

ACCACGATTATTGATACCATTAAGAAATCAAAAGCACCTGTGTTATTTGTTGAAACCAGT     900
T  T  I  I  D  T  I  K  K  S  K  A  P  V  L  F  V  E  T  S

GTCGATAAACGTAGTATGGAACGGGTCTCAAAAGAAGTGAAACAGCCAATTTACGATACA     960
V  D  K  R  S  M  E  R  V  S  K  E  V  K  Q  P  I  Y  D  T

CTTTTCACAGACTCCCTTGCCAAAGAAGGAACAGAAGGCGATACGTACTACAGCATGATG    1020
L  F  T  D  S  L  A  K  E  G  T  E  G  D  T  Y  Y  S  M  M

AACTGGAATTTAACAAAAATCCATGATGGCTTAATGAGTAAATAAATAATAAAGAAGAAA    1080
N  W  N  L  T  K  I  H  D  G  L  M  S  K  *
```

Figure 4.1. Nucleotide sequence of GP19, and the inferred amino acid sequence. The putative Shine Dalgarno (SD) sequence is double underlined. The sequence used for probing Southern and northern blots is underlined.

4.2. Nucleic acid and protein sequence analysis.

4.2.1. Open reading frames.

The partial nucleotide sequence of pGP19 was analysed for open reading frames (ORFs) using the PCGENE package (Intelligenetics). Three ORFs were identified, and are listed in table 4.1. in order of size.

Table 4.1. Open reading frames of pGP19.

Start codon.	Stop codon.	ORF length.	Peptide length.
139	1062	924 bp	308 aa
442	1062	621 bp	207 aa
838	1062	225 bp	75 aa

The program identifies open reading frames by looking for potential start sites (ATG), then reading through until a stop codon (TAA, TAG, TGA) is reached. In the three ORFs identified above, only the largest is likely to be the true ORF, the smaller two represent the software interpreting methionine codons within the largest ORF as start codons. A potential ribosome binding site (GGAGGA) occurs 9-14 bp upstream of the start codon at position 139. Because of time restrictions, approximately 700 bp of nucleic acid material at the 3'-end of the cloned insert were not sequenced. Earlier experiments had indicated that the open reading frame of the cloned immunoreactive protein was within the 5'-half of the insert, and that no reactive protein could be detected during expression from the unsequenced region of the insert (section 3.5.).

4.2.2 Analysis of the deduced amino acid sequence.

Translation of the open reading frame detected in section 4.2.1. revealed a 308 amino acid peptide with a predicted molecular weight of 34,768 Da and an isoelectric point of 5.04. The full protein sequence is shown in figure 4.1. This protein was taken to be the expression product of pGP19 identified in section 3.5. and was given the name EfaA (*Enterococcus faecalis* antigen A). The nucleic acid sequence of *efaA* has

been deposited in the GenBank and EMBL databases under the accession number U03756.

Further analysis using the PCGENE package revealed that EfaA is hydrophilic except for residues 1-19. The hydrophilicity profile of EfaA is shown in figure 4.2. EfaA as a whole has an average hydropathy index of -5.27 (hydrophilic) whereas residues 1-19 have an index of 14.78 (hydrophobic) (Kyte and Doolittle, 1982; see also appendix A). Residues 2-21 were predicted to form a transmembrane α-helix, according to the method of Rao and Argos (1986). Analysis of the peptide sequence with the PROSITE package revealed the presence of a potential signal peptidase cleavage site (LAAC) at residues 17-20. This sequence corresponds to the consensus sequence Leu(Val,Ile)-Ala(Ser,Thr,Gly)-Gly(Ala)-Cys (von Heijne, 1989) for cleavage by signal peptidase II.

This suggests that the first 19 amino acids of EfaA comprise a leader sequence, which is cleaved before the mature protein is formed. The mature protein would be hydrophilic, and have a molecular weight of 32,701.

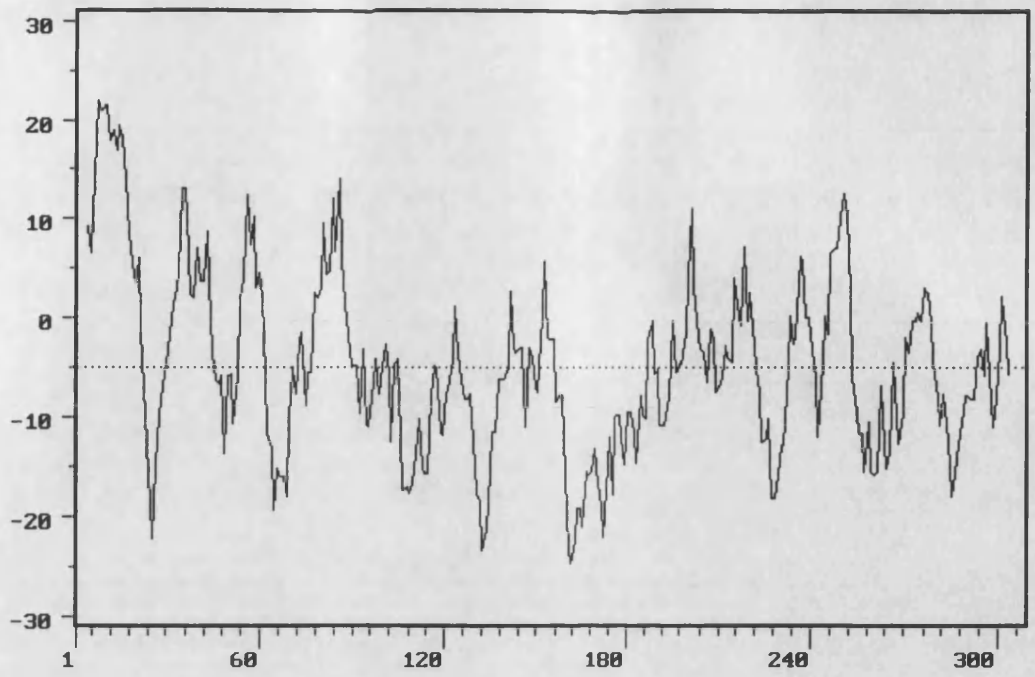

Figure 4.2. Hydropathic index of EfaA, computed by the Kyte-Doolittle 'SOAP' package using an interval of 9 amino acids.

4.2.3. Nucleic acid and protein sequence analysis: Comparison with databases.

The nucleotide sequence of *efaA* and the amino acid sequence of EfaA were analysed for similarity with other sequences in the GenBank and EMBL databases. The comparisons were performed using the GCG package on the SERC Seqnet facility at Daresbury, UK.

The FASTA program was used to compare the nucleotide sequence of *efaA* directly with all other nucleotide sequences in the GenBank and EMBL databases. The TFASTA program was used to compare the amino acid sequence of EfaA with nucleotide sequences in the databases translated in all six reading frames. The two programs together give an indication of homology with sequences deposited in the databases at the nucleic acid and amino acid level, respectively.

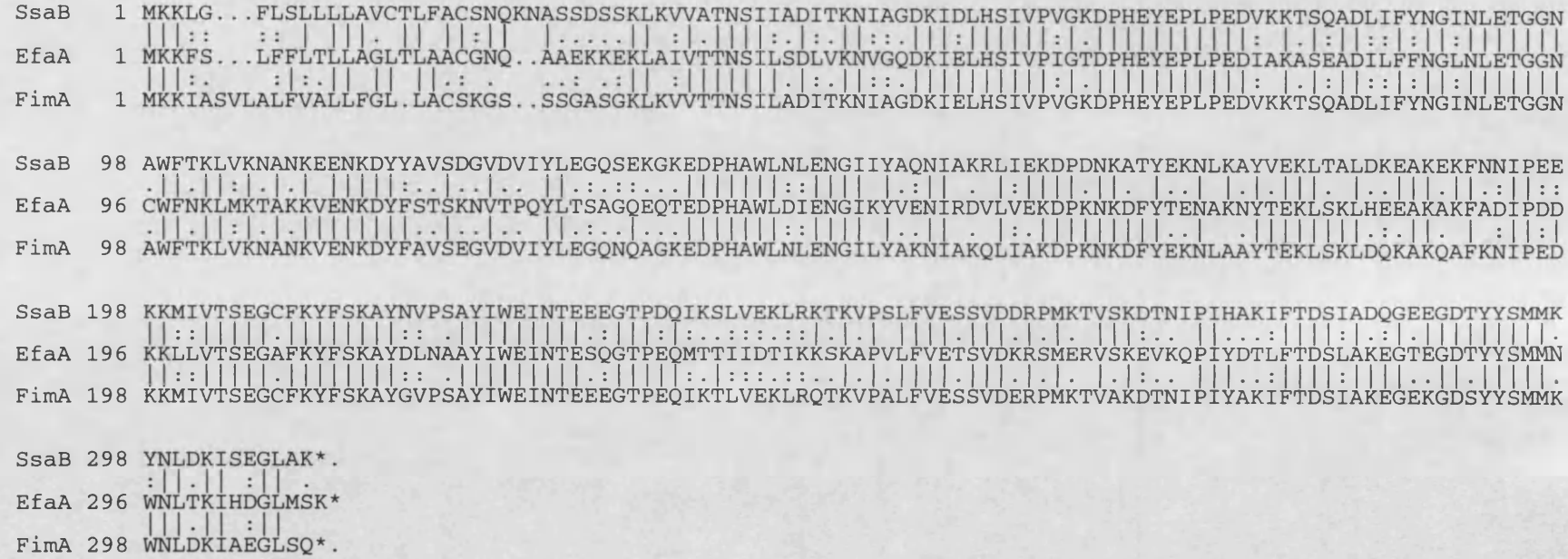

Fig 4.3 Sequence alignment of amino acid sequence of EfaA with SsaB (73% similar) and FimA (73% similar).
Sequence alignment was performed using the GAP program, part of the GCG package.

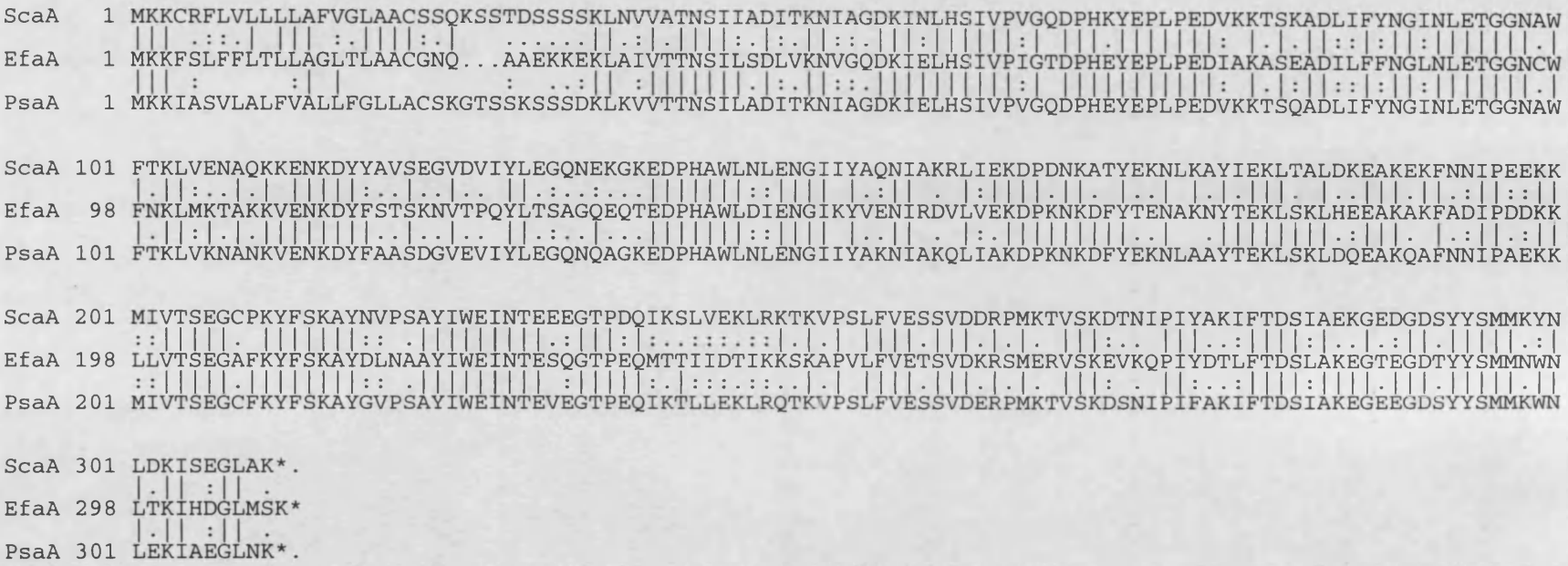

Fig 4.4. Sequence alignment of amino acid sequence of EfaA with ScaA (70% similar) and PsaA (72.5% similar). Sequence alignment was performed using the GAP program, part of the GCG package.

```
EfaA  MKKFSLFFLTLLAGL-TLAACGNQAAEKK---EKLAIVTTNSILSDLVKNVGQDKIELHSIVPIGTDPHEYEPLPEDI
FimA  MKKIASVLALFVALLFGLLACSKG-SSS-GASGKLKVVTTNSILADITKNIAGDKIELHSIVPVGKDPHEYEPLPEDV
PsaA  MKKIASVLALFVALLFGLLACSKG-TSSKSSSDKLKVVTTNSILADITKNIAGDKIELHSIVPVGQDPHEYEPLPEDV
ScaA  MKKC-RFLVLLLLAFVGLAACSSQKSSTDSSSSKLNVVATNSIIADITKNIAGDKINLHSIVPVGQDPHKYEPLPEDV
SsaB  MKKL-GFLSLLLLAVCTLFACSNQKNAS-SDSSKLKVVATNSIIADITKNIAGDKIDLHSIVPVGKDPHEYEPLPEDV
      ***    .  ..     * **.          ** .*.****..*. **.. ***.******.* *** *******.

EfaA  AKASEADILFFNGLNLETGGNCWFNKLMKTAKKVENKDYFSTSKNVTPQYLTSAGQEQTEDPHAWLDIENGIKYVENI
FimA  KKTSQADLIFYNGINLETGGNAWFTKLVKNANKVENKDYFAVSEGVDVIYLEGQNQAGKEDPHAWLNLENGILYAKNI
PsaA  KKTSQADLIFYNGINLETGGNAWFTKLVKNANKVENKDYFAASDGVEVIYLEGQNQAGKEDPHAWLNLENGIIYAKNI
ScaA  KKTSKADLIFYNGINLETGGNAWFTKLVENAQKKENKDYYAVSEGVDVIYLEGQNEKGKEDPHAWLNLENGIIYAQNI
SsaB  KKTSQADLIFYNGINLETGGNAWFTKLVKNANKEENKDYYAVSDGVDVIYLEGQSEKGKEDPHAWLNLENGIIYAQNI
       *.* **..*.**.******* ** **.  *.* *****.. *  *   ** .  .   *******..**** *  **

EfaA  RDVLVEKDPKNKDFYTENAKNYTEKLSKLHEEAKAKFADIPDDKKLLVTSEGAFKYFSKAYDLNAAYIWEINTESQGT
FimA  AKQLIAKDPKNKDFYEKNLAAYTEKLSKLDQKAKQAFKNIPEDKKMIVTSEGCFKYFSKAYGVPSAYIWEINTEEEGT
PsaA  AKQLIAKDPKNKDFYEKNLAAYTEKLSKLDQEAKQAFNNIPAEKKMIVTSEGCFKYFSKAYGVPSAYIWEINTEVEGT
ScaA  AKRLIEKDPDNKATYEKNLKAYIEKLTALDKEAKEKFNNIPEEKKMIVTSEGCPKYFSKAYNVPSAYIWEINTEEEGT
SsaB  AKRLIEKDPDNKATYEKNLKAYVEKLTALDKEAKEKFNNIPEEKKMIVTSEGCFKYFSKAYNVPSAYIWEINTEEEGT
         *. *** **  *  *   * ***. *.  **  * .** .**..*****  ******* . .********* .**

EfaA  PEQMTTIIDTIKKSKAPVLFVETSVDKRSMERVSKEVKQPIYDTLFTDSLAKEGTEGDTYYSMMNWNLTKIHDGLMSK
FimA  PEQIKTLVEKLRQTKVPALFVESSVDERPMKTVAKDTNIPIYAKIFTDSIAKEGEKGDSYYSMMKWNLDKIAEGL-SQ
PsaA  PEQIKTLLEKLRQTKVPSLFVESSVDERPMKTVSKDSNIPIFAKIFTDSIAKEGEEGDSYYSMMKWNLEKIAEGL-NK
ScaA  PDQIKSLVEKLRKTKVPSLFVESSVDDRPMKTVSKDTNIPIYAKIFTDSIAEKGEDGDSYYSMMKYNLDKISEGL-AK
SsaB  PDQIKSLVEKLRKTKVPSLFVESSVDDRPMKTVSKDTNIPIHAKIFTDSIADQGEEGDTYYSMMKYNLDKISEGL-AK
      *.*. .... ....* * ****.*** *.*  *.*. . **   .****.*  *  **.*****. ** ** .**  .
```

Fig 4.5. Multiple sequence alignment of EfaA, FimA, PsaA, ScaA and SsaB, generated by CLUSTALV. Identical residues are indicated by (*), conserved residues are indicated by (.). Homology over all five sequences is 51.3% (158 residues). Similarity over all five sequences is 70%.

EfaA was found to possess significant homology with four previously cloned proteins as shown in table 4.2. Three of these proteins have adhesive functions. FimA of *S. parasanguis* and SsaB of *S. sanguis* 12 appear to be salivary adhesins whilst ScaA mediates coaggregation of *S. gordonii* with other oral bacteria such as *Actinomyces naeslundii.* No function has yet been ascribed to PsaA of *S. pneumoniae.*

Table 4.2. Cloned proteins with homology to EfaA.

Protein	Species	Amino acid	Nucleic acid	
FimA	*S. parasanguis*	60.5%	61.8%	Fenno *et al* (1989)
SsaB	*S. sanguis* 12	57.7%	62.7%	Ganeshkumar *et al* (1991)
PsaA	*S. pneumoniae*	60.5%	61.2%	Sampson *et al* (1994)
ScaA	*S. gordonii*	55.5%	60.4%	Kolenbrander (unpub)

Homology values were calculated by the FASTA and TFASTA programs. Individual sequence alignments were generated using the GAP program, which also calculated similarity values for the alignments. The alignments are shown in figures 4.3. and 4.4. Similarity values account for conserved amino acid substitutions rather than just identical residues. The values calculated for similarity to EfaA were: FimA, 73%; SsaB, 73%; PsaA 72.5%; and ScaA, 70%. The sequences were also aligned using the multiple sequence alignment package ClustalV, which calculated an overall similarity between the 5 proteins as 70% (figure 4.5.).

The sequences can be found under the following GenBank accession numbers: FimA, M26130; SsaB, M37189; PsaA, L19055; ScaA, L11577.

4.2.4. Analysis of DNA regions flanking efaA.

DNA flanking the *fimA*, *psaA* and *scaA* genes share a number of features, including flanking reading frames both upstream and downstream of their putative adhesin genes. The upstream reading frames encode proteins of approx 29.9 kDa, which are predicted to be extremely hydrophobic. Analysis of the 139 bases upstream of the ATG start codon of *efaA* within pGP19- by translation of the three potential

reading frames revealed that frames 1 and 2 contained numerous stop codons, whilst frame 3 had none. Analysis of the amino acid translation of frame 3 revealed some homology to the open reading frames upstream of the *fimA*, *psaA* and *scaA* genes. An alignment of the homologous regions is shown in figure 4.6.

```
EfaA  ----SGATIVLTAALFFLLAFFFSPKKGLVFVNREKEMEESTNEKI
FimA  LNIAVGSCIVLTSAVFFLISFFIAPKQ------RKNKHALSSH---
PsaA  FNIAVGSCIVLTSAIFFLISFFIAPKQ------RKNKHALSPH---
ScaA  FNVAAGSSIVLTSALIFLVSFFIAPKQ------RYLKRKVK-----
           *. ****.*..**..**..**.       *
```

Figure 4.6. ClustalV alignment of the regions upstream of the *efaA*, *fimA*, *psaA* and *scaA* genes. Identical residues are denoted (*), conserved residues are denoted (.)

The translation continues to a stop codon (TAG) at base 150, thus the reading frame overlaps with that of *efaA* which begins at base 139, in frame 1. Within the *fimA*, *psaA* and *scaA* flanking sequences, the 29.9 kDa protein reading frames stop 12, 12 and 22 bp upstream of the putative adhesin gene start sites, respectively, with no transcriptional terminators between the genes. This suggests that in each case, the genes could be transcribed as part of a polycistronic message. The similarity observed suggests a common origin for these genes, however, *efaA* homology with the upstream reading frames of *fimA*, *psaA* and *scaA* ceases at base 93, and no stop codon is present until several bases into the *efaA* reading frame. Because of the lack of sequence data, it is uncertain whether or not the 29.9 kDa hydrophobic protein is expressed in *E. faecalis*. No open reading frames are reported in the 1 kb of cloned DNA upstream of the *ssaB* gene of *S. sanguis* 12, however, translation of the available sequence into a hypothetical peptide reveals a very high level of homology with the 29.9 kDa proteins upstream of *fimA*, *psaA* and *scaA*. This peptide is apparently not expressed, since it is not within an open reading frame, so similarly to the situation in *E. faecalis*, the *ssaB* operon of *S. sanguis* 12 appears to have diverged from *fimA*, *psaA* and *scaA*.

Northern blot analysis of *S. parasanguis* cellular RNA showed that *fimA* is transcribed on a 3.2 kb message (Fives-Taylor *et al*, 1991), similarly, *psaA* has been

shown to be transcribed on a 3 kb message (Sampson *et al*, 1994). These data are in keeping with the proteins being transcribed as a polycistronic message. Although no function has as yet been positively ascribed to these flanking genes, Andersen *et al*, (1994) who have sequenced over 6 kb of DNA flanking *scaA*, have noted similarities between the scaA operon and periplasmic binding protein systems in Gram-negative bacteria (discussed in section 4.5.19.). Such systems have also been reported in Gram-positive species (Alloing *et al*, 1990) suggesting that ScaA may be part of a lipoprotein-dependant membrane transport system. The evidence from the *ssaB* operon clearly indicates that the hydrophobic 29.9 kDa protein is not an essential requirement for saliva binding, however it may serve some other purpose in relation to the adhesins. A principal difference between FimA and SsaB is that FimA is present in surface fimbriae of *S. parasanguis*, whereas SsaB appears to be associated with short fibrils (Ganeshkumar *et al*, 1988), with fibrils being defined as structures extending from the cell surface which have a defined length, but no consistent width, probably because they clump (Handley *et al*, 1984). The manufacture of fimbriae in *S. parasanguis* has been shown to involve the interaction of a number of proteins (Fives-Taylor *et al*, 1991), so it may be that in losing the ability to express the 29.9 kDa protein, *S. sanguis* 12 has lost the ability to include SsaB in long fimbriae, and instead, through some altered process expresses its adhesin in a different form. Some strains of *Enterococcus faecalis* have been shown to possess fimbriae (Handley and Jacob, 1981). The number of *E. faecalis* fimbriate cells in a population was shown to vary according to growth phase, and the pattern of appearance and disappearance of fimbriate cells varied according to the *E. faecalis* strain studied. Electron microscopy has shown that approximately 35% of log-phase *E. faecalis* EBH1 cells possess fimbriae, but that this figure drops to around 5% in stationary phase cells. Immunogold labelling of *E. faecalis* EBH1 cells using antiserum raised against the 37 and 40 kDa antigens demonstrated that the antigens were present on the cell surface, but like SsaB, were not associated with fimbriae (Shorrock, 1990).

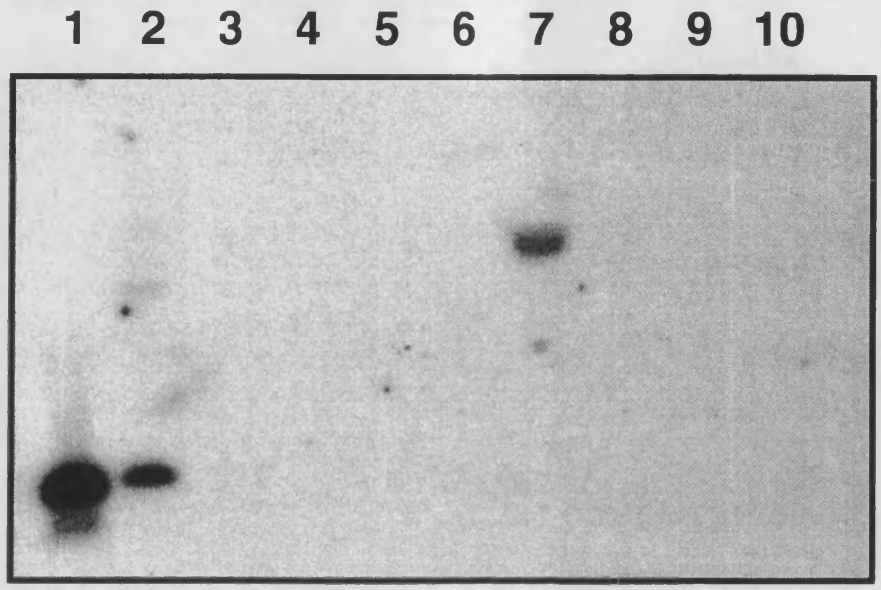

Figure 4.7. Southern blot analysis of *Hin*dIII digested genomic DNA from various enterococcal and streptococcal strains, probed with a 0.4kb *Hin*dIII fragment of *E. faecalis* EBH1 *efaA*. The blot was washed in 0.1x SSC at 68°C for 20 minutes.

Lane 1, *E. faecalis* EBH1; lane 2, *E. faecalis* NCTC5957; lane 3, *S. pneumoniae* (blood isolate); lane 4, *S. pneumoniae* (sputum isolate); lane 5, *S. parasanguis* FW213; lane 6, *S. gordonii* Challis; lane 7, *E. faecium* ATCC10541; lane 8, *S. salivarius* NCTC8618; lane 9, *S. bovis* Beecham 5240453; lane 10, *S. milleri* NCTC7863.

4.3. Southern Analysis.

Southern analysis was performed to determine whether genes homologous to *efaA* were present in other streptococci (See table 4.3.). Chromosomal DNA was digested to completion with *Hin*dIII, separated on an agarose gel, and the fragments transferred to a nitrocellulose filter by capillary flow, as described by Southern *et al* (1975). Blots were probed with a 405 bp internal *Hin*dIII fragment, labelled with ^{32}P. The sequence of this probe is underlined in figure 4.1.

Table 4.3. Bacterial strains analysed by Southern blotting.

Strain.	Source.
E. faecalis EBH1	Endocarditis isolate, B'ham Heartlands Hosp.
E. faecalis (Docker 10482)	Endocarditis isolate, B'ham Heartlands Hosp.
E. faecalis (Phillips 6546)	Endocarditis isolate, B'ham Heartlands Hosp.
E. faecalis (LJ)	Endocarditis isolate, B'ham Heartlands Hosp.
E. faecalis (MHC)	Endocarditis isolate, B'ham Heartlands Hosp.
E. faecalis (777)	Endocarditis isolate, B'ham Heartlands Hosp.
E. faecalis (SFBA)	Endocarditis isolate, B'ham Heartlands Hosp.
E. faecalis v. zymogenes	NCTC 05957
Strep. milleri	NCTC 07863
Strep. mutans	NCTC 10449
Strep. salivarius	NCTC 08618
E. faecium	ATCC 10541
Strep. bovis	SmithKline Beecham 5240453
Strep. mitis	Clinical isolate, Newcastle Dental School
Strep. parasanguis FW213	Dr P.S. Handley, Manchester University
Strep. gordonii challis	Dr P.S. Handley, Manchester University
Strep. pneumoniae (blood isolate)	Dr. I.D. Farrell, B'ham Heartlands Hosp.
Strep. pneumoniae (sputum isolate)	Dr. I.D. Farrell, B'ham Heartlands Hosp.

The labelled probe reacted at high stringency with an approx 0.4 kb fragment from all *E. faecalis* strains tested, and hybridised weakly to an approximately 3 kb *Hin*dIII fragment of *E. faecium* ATCC10541 (figure 4.7.). This band faded, but was

not completely removed, by high stringency washes. No other strains reacted, even under conditions of low stringency.

4.4. Bacterial adherence to hydrocarbon assay (BATH assay).

Computer analysis of the amino acid sequence of EfaA predicted that the protein is very hydrophilic. Previous experiments by Lambert *et al*, (1990) have shown that expression of the 37 and 40 kDa antigens is regulated by serum. In order to determine the effects of serum-supplemented growth upon hydrophilicity of *E. faecalis* EBH1, BATH assays were performed. The BATH assay provides a measure of the surface hydrophilicity of a bacterium by measuring its association with a hydrophobic solvent (hexadecane). The reduction in OD_{450} of an aqueous bacterial suspension after mixing with hexadecane is proportional to the hydrophobicity of the cells. Results are shown as the OD_{450} of the hexadecane-extracted cells, expressed as a percentage of the OD_{450} of the original suspension. Thus the higher the percentage, the more hydrophilic the cells. In order to determine the effect of serum in the growth medium on EBH1 hydrophobicity, BATH assays were performed on cells grown in yeast extract, and in yeast extract supplemented with foetal calf serum (FCS). For each growth medium, three cultures were grown, and for each culture the experiment was performed in triplicate. Means were compared by unpaired students t-test.

Table 4.4. BATH assay of *E. faecalis* EBH1 cells in different culture media.

1% yeast extract.	1% yeast extract plus FCS.
Individual means:	
37.6% (n=3)	60.5% (n=3)
50.5% (n=3)	60.1% (n=3)
37.8% (n=3)	68.9% (n=3)
Pooled means (± std deviation):	
42(±7.4)% (n=9)	63.2(±4.9)% (n=9)

The results indicate that addition of serum significantly increased the hydrophilicity of *E. faecalis* EBH1 cells ($p < 0.05$).

4.5. Discussion.

4.5.1. Plasmid pGP19 open reading frame.

Computer analysis of the complete nucleotide sequence of pGP19 revealed a single 924 bp open reading frame, encoding a 308 amino acid protein, with a predicted molecular weight of 34,768. As this was the only protein encoded by the cloned DNA, it was assumed that this represents the single band seen when GP19 was induced with IPTG. This is further supported by the presence of the polypurine sequence GGAGGAA, which agrees with the consensus sequence GGAGGA (Shine and Dalgarno, 1974) which is complementary to a 3' segment of prokaryotic 16S RNA necessary for ribosome binding to mRNA ahead of the start codon ATG.

4.5.2. Post-translational modification.

The predicted molecular weight of EfaA is somewhat smaller than that of the expressed peptide, as calculated by SDS-PAGE. The rate of migration of denatured proteins in a denaturing SDS-PAGE gel is assumed to be a function solely of its molecular weight, since all secondary structure is disrupted by boiling in the presence of SDS and mercaptoethanol, and the primary chain is then coated with negatively-charged dodecyl sulphate molecules. However, this method provides, at best, only an estimate of the true molecular weight of a mature protein. It is not unusual for the apparent molecular weight of a protein as determined by SDS-PAGE to differ from that calculated from its sequence data (Sela *et al*, 1993; Ichikawa *et al* 1994). Indeed a similar discrepancy is seen in the predicted and apparent weights of some of the EfaA homologues listed in section 4.2.3. (Fenno *et al*, 1989; Ganeshkumar *et al* 1991)

The rate of protein migration through a polyacrylamide gel can be retarded by post-translational modifications to the polypeptide backbone such as glycosylation. Thus the apparent molecular weight of a glycosylated protein estimated by SDS-PAGE is not a true reflection of the weight of the peptide chain. On this basis, it is suggested that EfaA may undergo some post-translational glycosylation. This is in agreement with previous work by Aitchison *et al* (1986), which used lectins in ligand blotting and periodate treatment of western blots, and suggested that the 37 and 40 antigens were glycosylated. Reports of glycosylation of bacterial proteins are rare, however those reported have included surface proteins which are thought to interact with eukaryotic receptors (Sambri *et al*, 1992; Hirt *et al*, 1993).

4.5.3. Analysis of pGP19- for promoter sequences.

Weak expression of EfaA from pGP19- in the absence of inducer prompted the theory that some promoter elements may have been cloned along with structural genes in pGP19 (See section 3.7.2.). Computer analysis of the nucleotide sequence of pGP19- revealed no potential promoter sequences upstream of the ATG start codon at base 139. The lack of any such potential promoter lends support to the theory that expression in the absence of inducer is due to random A-T rich sequences of *E. faecalis* DNA being recognised as promoters by *E. coli* RNA polymerase (see section 3.7.2.). It is noteworthy that cloned PsaA of *S. pneumoniae*, with which EfaA shares substantial homology, also exhibits significant expression from its cloning vector in the absence of inducer, despite there being no *S. pneumoniae* promoter elements in the cloned fragment (Sampson *et al*, 1994). This also has been attributed to random *E. coli* promoter-like sequences occurring in the A-T rich *S. pneumoniae* chromosome.

4.5.4. Hydrophilicity analysis of EfaA.

The Kyte and Doolittle computer software (SOAP) analyses the hydrophobicity and hydrophilicity of a protein according to its amino acid sequence, displaying the results graphically (Kyte and Doolittle, 1982). Such analysis also gives rise to a GRAVY score (GRAnd AVerage hydropathY) which is an indicator of the general hydrophilic or hydrophobic nature of the protein. The Kyte-Doolittle plot for EfaA is shown in figure 4.2. The GRAVY score of EfaA is -5.27, indicating that the protein is generally hydrophilic, and soluble in nature. Hopp and Woods (1981) deduced that the major antigenic determinants of a surface protein are most commonly those subsequences of greatest hydrophilicity, since these sections are exposed most prominently to the aqueous medium surrounding the organism. Furthermore, antigenicity can be correlated to segmental flexibility along the peptide chain (Van Regenmortel, 1986), with those regions of most pronounced flexibility combined with hydrophilicity being the most likely antigenic determinants. Computer analysis using the FLEXPRO package (PCGene, Intelligenetics), suggests that EfaA contains a large number of potential hydrophilic epitopes and a large proportion of 'flexible' residues as compared to 'rigid' residues, which would make it antigenic. This supports the evidence that EfaA represents one, or both, of the 37 and 40 kDa antigens of *E. faecalis*, which have been shown to elicit a strong antibody response both in humans, and in rabbits (Shorrock *et al*, 1990; Lambert *et al*, 1990).

If EfaA does represent the 37 and 40 kDa antigens, then its expression should be enhanced by growth in serum-supplemented media, and be accompanied by an increase in cell surface hydrophilicity, since the protein is very hydrophilic. BATH assays did indeed indicate that growth of *E. faecalis* cells in serum-supplemented media was associated with increased cell hydrophilicity. These data are in agreement with similar work by Guzman *et al*, (1991a), and further support the link between EfaA and the 37 and 40 kDa antigens of *E. faecalis*.

4.5.5. Location of EfaA.

Since EfaA is hydrophilic and sufficiently surface exposed to elicit a strong antibody response, then it must be assumed that a specific mechanism exists to attach the protein to the cell surface. Such an attachment must prevent loss of the protein, whilst allowing the hydrophilic backbone to extend into the aqueous medium to exercise its specific function. Examining the Kyte-Doolittle hydrophilicity profile of the protein may give an indication of the nature of this attachment. The exception to the hydrophilicity of the primary chain of EfaA is the N-terminal 19 amino acids. Taken separately, these 19 amino acids have a GRAVY score of 14.78 (very hydrophobic) leaving the remaining 289 residues with a GRAVY score of -6.59 (hydrophilic). This hydrophobic region could have a membrane anchor function, the hydrophobic region of the protein being associated with the lipid bilayer of the cell membrane. Alternately, the hydrophobic region may represent a signal peptide, ultimately cleaved by a signal peptidase to form the mature lipoprotein.

Many surface proteins of Gram-positive cocci have been shown to be held at the cell surface by means of such a hydrophobic anchor (Fischetti *et al*, 1991; Schneewind *et al*, 1991; 1992). These proteins are characterised as having a C-terminal run of up to seven basic amino acids. Immediately N-terminal to this is a 15-22 residue hydrophobic region, the putative membrane anchor, followed by highly conserved hexapeptide with the consensus sequence LPXTGX (Fischetti *et al*, 1991), and a wall-associated region. Disruption of the C-terminus of one of these characterised surface proteins, CshA of *S. gordonii*, caused the truncated product to be secreted into the extracellular medium (McNab and Jenkinson, 1992), providing confirmation that this region is indeed vital for attachment to the cell surface. Furthermore, minor alterations to either the LPXTGX motif or to the charged C-terminal residues result in incorrect processing (Schneewind *et al*, 1992), indicating that these regions play central roles in surface protein localisation.

This suggests a potential membrane anchor role for the hydrophobic region of EfaA. Clearly, however, the putative anchor region of EfaA is heterologous to those of the characterised Gram-positive surface proteins discussed above, since the hydrophobic region is N-terminal rather than C-terminal, and no sequence homologous to the LPXTGX hexapeptide is seen. It is therefore proposed that EfaA is attached to the cell surface by some other means.

Examination of the N-terminal sequence of EfaA reveals the amino acid motif L-A-A-C at residues 17-20. This sequence corresponds to the consensus sequence for cleavage by signal peptidase II, the enzyme which recognises and processes lipoprotein precursors in prokaryotes. This suggests that EfaA may itself be a lipoprotein, attached to the cell surface not by association of hydrophobic amino acids, but by means of an N-terminal lipo-amino acid anchor (Gilson *et al*, 1988).

4.5.6. Bacterial lipoproteins.

In prokaryotes, lipoproteins are translated as precursor proteins (prolipoproteins) containing an N-terminal signal peptide which is eventually cleaved by a specific lipoprotein signal peptidase (signal peptidase II). The lipoprotein signal peptide is characterised by a basic N-terminus, a hydrophobic core and a cleavage site. Since primary sequence homology among the signal sequences of prolipoproteins is low (other than within the cleavage site) it is likely that the conformation of the signal peptide, particularly the formation of an α-helix, is the conserved factor (Engelman and Steitz, 1981; Chou and Kendall, 1990). Within the cleavage site, the consensus sequence (L,V,I)-(A,S,T,G)-(G,A)-C is observed, requiring at least one match in the first two positions, and precise matches to the final two (von Heijne, 1989).

The signal sequence performs many functions, probably involving interactions with many cellular components common to both lipoprotein and non-lipoprotein signal peptides (von Heijne, 1989). Gierasch (1989) proposed a scheme in which SecA, a cytoplasmic protein, binds to the signal sequence of the growing polypeptide

chain at the ribosome, and targets the protein to the cell membrane. This stage may also involve other factors such as SecB and 'trigger factor'. The prolipoprotein would then bind to the membrane, in a step mediated by PrlA, a membrane protein receptor, and then be translocated across the membrane before finally being processed into a mature lipoprotein.

The hydrophobic region of the signal peptide allows association of the precursor with the lipid bilayer of the cytoplasmic membrane, presenting the cleavage site (LAAC) to the membrane bound processing enzymes. The site is first recognised by prolipoprotein glyceryl transferases which modify the cysteine residue by transfer of glyceride to its sulphydryl group. The leader peptide is then cleaved by signal peptidase II, and the N-terminal cysteine amino group is acylated, forming the final lipoprotein (Hayashi and Wu, 1990; Dawes and Sutherland, 1992).

Signal peptidases are integral membrane proteins which reside in the plasma membrane. The active site is probably located within, or just external to, the lipid bilayer (Dalbey and von Heijne, 1992). Thus the signal peptide must act to present the cleavage site of the prolipoprotein accurately to the signal peptidase. Small alterations in the basic N-terminus and/or the length of the hydrophobic region can cause a shift of the signal peptide within the plasma membrane, greatly affecting processing (Nothwehr and Gordon, 1990; Chou and Kendal, 1990). Such alterations can result in the mutant prolipoprotein remaining permanently anchored to the cytoplasmic membrane via its signal peptide. Despite this lack of processing, such mutants can still function correctly (Fikes and Bassford, 1987), which prompts some debate as to the function of the lipid anchor. Clearly it exists to maintain the functional molecule within the vicinity of, or attached to, the cell, but the question remains as to why a lipid anchor, and not a hydrophobic amino acid anchor, as seen with other surface proteins (Fischetti *et al*, 1991)?

4.5.7. Evidence that EfaA may be a lipoprotein.

The basic N-terminus, hydrophobic α-helix core and cleavage site of the EfaA signal peptide all correspond to the requirements of the prolipoprotein processing enzymes of prokaryotes. Furthermore, there are well documented cases of proteins with signal peptides homologous to that of EfaA being confirmed as lipoproteins, by incorporation of [^{3}H] labelled palmitate, such as SarA of *S. gordonii* (Jenkinson, 1992), SsaB of *S. sanguis* 12 (Ganeshkumar *et al*, 1993) and ScaA of *S. gordonii* (Andersen *et al*, 1994). Ganeshkumar et al (1993) further demonstrated by site directed mutagenesis that a cysteine at amino acid 20 of SsaB was required for the incorporation of the radiolabelled palmitate. This is in agreement with Nielsen and Lampen (1982) who first demonstrated the presence of glyceride-cysteine thioether lipoproteins in Gram-positive bacteria.

This evidence would suggest that EfaA is translated as a prolipoprotein, then modified at the plasma membrane by a glyceryl transferase and signal peptidase II. This would reveal a 32,701 Da hydrophilic mature protein, with an N-terminal cysteine covalently attached to a lipid moiety. This mature form of EfaA would be a surface-exposed protein, anchored to the cell membrane by its N-terminal lipid.

4.5.8. Southern analysis of streptococcal strains.

In Southern analysis, a radiolabelled *efaA* probe failed to hybridise to DNA from any streptococci (see table 4.3.). Other than *E. faecalis,* only one strain, *Enterococcus faecium*, was reactive with the probe. The lack of hybridisation with streptococcal strains is not surprising. Homology between *efaA* and the adhesin genes from other streptococci (60.4%-62.7%) is significant in terms of the similarity between the translated gene products, but clearly not sufficient to allow hybridisation of the nucleic acids at the stringencies used, since a final wash at 50°C in 2 x SSC

would require approximately 75% homology to the probe (Mason and Williams, 1985). It is therefore apparent that the streptococci tested do not possess *efaA*-like genes other than the adhesins mentioned in section 4.2.3. Southern analysis by Andersen *et al*, (1993) using *scaA* and *ssaB* probes failed to show hybridisation with *E. faecalis* DNA, which is in agreement with the results presented here.

The reactivity of the *efaA* probe with *E. faecium* reflects the similarity between the species. The match is clearly not perfect since the band is feint, and shows restriction fragment length polymorphism. Furthermore, antiserum reactive with the 37 and 40 kDa antigens of *E. faecalis* did not recognise any surface antigens of similar molecular weight in *E. faecium* (Aitchison *et al*, 1987), indicating that the gene products have different antigenic epitopes.

4.5.9. Homology analysis by comparison with databases.

Comparison of the primary amino acid sequence of EfaA with the GenBank and EMBL databases revealed homology with a group of previously cloned proteins from streptococci (FimA, SsaB, ScaA and PsaA, see table 4.2.). Homology with EfaA ranges from 55.5% to 60.5%, with greatest divergence at the N-terminal ends, which is in keeping with this region being a signal peptide. It has been established that the N-terminus of SsaB is a signal sequence, cleaved at Cys-20 by signal peptidase II (Ganeshkumar *et al*, 1993), and so it is likely that the N-termini of FimA, PsaA and ScaA are similar signal sequences. Homology studies and Southern hybridisation have shown that EfaA is not so closely related to FimA, SsaB, ScaA and PsaA as they are to each other (80-90%). Nevertheless, the high degree of homology between EfaA and this group of proteins indicates that a great deal of information about the function of EfaA can be gained by an examination of their properties.

4.5.10. SsaB of Streptococcus sanguis.

Members of the group of bacteria derived from *Streptococcus sanguis* (including *S. sanguis* 12, *S. gordonii* and *S. parasanguis*) are oral organisms that selectively adhere to the acquired salivary pellicle of the teeth. They also have the ability to bind other oral organisms, making them a major component (8 to 15%) of dental plaque (Gibbons and van Houte, 1975). Furthermore, this group of streptococci are a major cause of infective endocarditis (See section 1.1.1.).

Studies of the binding of wild type *S. sanguis* 12 to saliva-coated hydroxyapatite (SCHA) compared to a non-aggregating mutant suggested two separate means of binding to saliva (Morris and McBride, 1984). Morris et al (1985) characterised one of these mechanisms as being linked to fibrillar structures on the surface of *S. sanguis* 12. Monoclonal antibodies raised against these *S. sanguis* 12 fibrils inhibited adherence to SCHA (Morris et al, 1987). The second salivary adhesin was identified by Ganeshkumar *et al* (1988) by screening an *S. sanguis* 12 genomic library with antiserum reactive with *S. sanguis* 12 whole cells. The adhesin was found to be a 36 kDa protein which was present on the cell surface of both wild type, and the non-aggregating mutants of *S. sanguis* 12, possibly as densely packed short fibrils. Purified SsaB partially inhibited binding of wild type *S. sanguis* 12 and abolished binding of non-aggregating mutants. This confirmed the two site salivary binding model of Morris and McBride (1984), with non-aggregating mutants lacking the long fibrillar adhesin, but retaining SsaB. Nucleotide sequencing of *ssaB* revealed a 34,684 Da hydrophilic protein with a putative 19 amino acid signal peptide (Ganeshkumar *et al*, 1991), which was later confirmed as a lipoprotein (Ganeshkumar *et al*, 1993). The amino acid sequence of SsaB was found to be 87% homologous to FimA, a functionally similar protein from *S. parasanguis* (Fenno *et al*, 1989).

4.5.11. FimA of Streptococcus parasanguis.

Mutants of *S. parasanguis* FW213 (previously *S. sanguis*, Whiley *et al*, 1990) nonadherent to SCHA were characterised by Fives-Taylor and Thompson (1985) and found to lack fimbriae, which are present in the wild-type strain. Coaggregation with other oral bacteria was not affected. Antibodies raised against these fimbriae blocked adhesion of fimbriated cells to SCHA (Fachon-Kalweit *et al*, 1985), suggesting that fimbriae were directly involved in adhesion to components of saliva. The structural gene for an antigenic component of fimbriae was cloned and sequenced (Fives-Taylor *et al*, 1987; Fenno *et al*, 1989), and found to be a 36 kDa protein designated FimA. FimA was shown to have a high affinity for its substrate on the salivary pellicle, purified protein inhibited binding of *S. parasanguis* to SCHA, and displaced cells already bound to SCHA (Oligino and Fives-Taylor, 1993). Besides sharing sequence homology and function, both FimA and SsaB renatured as polymeric forms after purification, suggesting that both proteins may perform structural as well as adhesive functions. Polymeric forms of FimA were resistant to boiling in SDS and mercaptoethanol (Oligino and Fives-Taylor, 1993), whereas SsaB polymers were reduced to monomers by incubation with SDS at room temperature (Ganeshkumar *et al*, 1988). FimA is known to be associated with fimbria of *S. parasanguis*, so the suggestion is that it is a structural part of the fimbria as well as an adhesin. Such an appendage, with an adhesin present on each subunit, could allow strong adherence to the substrate (Oligino and Fives-Taylor, 1993). SsaB is not associated with the long fibrils seen on *S. sanguis* 12, however, immunogold labelling has shown the protein as a halo around the cell surface, possibly as densely packed short fibrils (Ganeshkumar *et al*, 1988).

4.5.12. ScaA of Streptococcus gordonii.

ScaA, in contrast to FimA and SsaB, mediates coaggregation with other oral organisms, rather than adhesion to saliva. *S. gordonii*, previously classified as

S. sanguis, coaggregates with *Actinomyces naeslundii*, another early coloniser of the oral cavity. Immunoblot analysis of *S. gordonii* using anti-*S. gordonii* serum, adsorbed with coaggregation-defective mutants, identified a 38 kDa surface protein of *S. gordonii* which appeared to mediate coaggregation with *A. naeslundii* (Kolenbrander and Andersen, 1990). The antiserum selectively inhibited coaggregation between *A. naeslundii* and *S. gordonii*, whilst not affecting any other *S. gordonii* coaggregations. Western blots probed with anti-ScaA antiserum, and Southern blots probed with the *scaA* gene both suggested the presence of a ScaA-like protein in a group of viridans streptococci including *S. sanguis*, *S. parasanguis*, *S. oralis*, *S. milleri* and *S. oralis*, all of which, except *S. parasanguis*, exhibited coaggregation with *A. naeslundii*, which could be reversed with anti-ScaA serum (Andersen *et al*, 1993). No hybridisation or cross reactivity was detected with *E. faecalis*. It would appear that ScaA, FimA and SsaB are a distinct class of adhesin proteins, and that many other viridans streptococci possess similar, as yet uncharacterised proteins.

4.5.13. PsaA of Streptococcus pneumoniae.

Streptococcus pneumoniae is a major respiratory pathogen, responsible for approximately 70% of cases of bacterial pneumonia. Pneumococci are common inhabitants of the human upper respiratory tract, present in up to 70% of the normal adult population. Community-acquired pneumococcal pneumonia is usually a sporadic disease in carriers with injury to pulmonary defence mechanisms resulting in reduced bacterial clearance (Roberts, 1990). The *S. pneumoniae* surface antigen PsaA was first reported by Russell *et al* (1990). Monoclonal antibodies recognised a 37 kDa protein present in 24 different types of *S. pneumoniae* but not in any of the 19 other genera tested. The genera tested were selected on the basis of their ability to cause lower respiratory tract disease, so no data on cross reactivity with FimA, SsaB or ScaA are available. Immunisation of mice with the 37 kDa protein was found to protect against *S. pneumoniae* challenge, indicating possible usefulness as a vaccine (Talkington *et al*,

1992). Sequence analysis of the *psaA* gene revealed 90.5% homology to FimA and 73.6% to SsaB (Sampson *et al*, 1994). No experimental evidence exists as to the function of PsaA, however, the significant sequence homology with previously cloned adhesins leads to the suggestion that PsaA could be involved in adherence to surfaces in the respiratory tract, and as such could have a significant role in pathogenesis.

4.5.14. Distribution of the streptococcal adhesins.

This group of proteins represents a distinct, conserved class of streptococcal cell surface adhesins. Southern blot analysis by Andersen *et al* (1993) showed the presence of *scaA*-like genes in a range of oral streptococci, however, the discovery of PsaA in *S. pneumoniae*, and EfaA in *E. faecalis* indicates that the proteins are not limited to those organisms present in the oral cavity. The lack of hybridisation and cross-reactivity seen with *E. faecalis* on western and Southern blots is probably due to the relatively low homology between ScaA and EfaA, as compared to FimA and SsaB. Western blots using an anti-*E. faecalis* serum previously showed no cross reactivity between the 37 and 40 kDa antigens and surface antigens of other streptococci, including *S. sanguis* (Aitchison *et al*, 1987). Similar work using monoclonal antibodies to PsaA failed to detect cross-reactivity with any species other than *S. pneumoniae*, although the species tested were chosen on the basis of their ability to cause respiratory infections, and so did not include oral streptococci (Russell *et al*, 1990).

Because of the significant homology between the proteins, it is assumed that they perform similar functions in their respective hosts, ie adhesion, or coaggregation with other organisms present in the same environment. Either of these two functions would represent a significant virulence factor.

4.5.15. Oral adhesion.

Whilst adhesion to the target tissue is a major factor in colonisation of the host, the establishment of an oral bacterial population is promoted by both intra and intergeneric coaggregation of the colonising organisms (Kolenbrander *et al* 1990). An adhesin molecule which performs both of these functions would give the pathogen a distinct advantage. It seems likely that SsaB functions both in coaggregation and adhesion, however, *S. parasanguis* does not coaggregate with actinomyces, so clearly FimA does not mediate coaggregation in the same way as SsaB and ScaA, but does function as a salivary adhesin. (Andersen *et al*, 1993; Oligino and Fives-Taylor, 1993). ScaA has been shown to mediate coaggregation of *S. gordonii* with actinomyces, but no data are available concerning its ability to bind to saliva. An examination of the distribution of the oral flora by Frandsen *et al*, (1991) found that whilst *S. sanguis* was present in early plaque (within 4 hours of cleaning teeth), *S. gordonii* was present only in mature plaque. This is in keeping with *S. sanguis* being able to bind to, and colonise the acquired salivary pellicle of newly cleaned teeth, with *S. gordonii* acting as a secondary coloniser, through coaggregation with other oral bacteria. *S. gordonii* has been shown to adhere to some components of saliva (Gibbons *et al*, 1991; Jenkinson *et al*, 1993), but the presence of other streptococci and lactobacilli in the oral cavity may prevent its early establishment (Loach *et al*, 1994).

The accretion of dental plaque is a complex phenomenon, involving many different bacterial surface components, which may act as specific or non-specific adhesins (Busscher *et al* 1992). Following initial adhesion, coaggregation appears to play a pivotal role in accretion and maintenance of dental plaque (Kolenbrander *et al* 1990). In general the early colonisers of freshly cleaned teeth, ie mainly streptococci and actinomyces, coaggregate with other early colonisers, and with fusobacteria. Late colonisers coaggregate almost exclusively with fusobacteria. Thus, coaggregation partners are temporally related with respect to formation of plaque (Kolenbrander *et al*, 1993). Early colonisation of the tooth surface is by direct attachment to the

acquired salivary pellicle, which covers teeth following cleaning. Initial binding of streptococci may involve salivary acidic proline rich proteins (Gibbons *et al*, 1991), or bacterial components such as cell wall fragments and membrane proteins, following which, intrageneric coaggregation helps to establish the early colonisers (Kolenbrander *et al* 1993).

Coaggregation among resident bacteria appears to be a characteristic unique to the oral ecosystem (Kolenbrander and London 1993), although recent reports suggest that the ability of *E. faecalis* to express aggregation substance, an adhesin which causes clumping of *E. faecalis* cells, can contribute to pathogenicity in endocarditis (Chow *et al*, 1993).

Enterococci are often found in the oral cavity, but their numbers are low, particularly when compared to species such as *S. sanguis*, *S. mitis*, *S. salivarius* and *S. mutans*. This may be due to evolutionary changes which have lead to a loss of the ability of enterococci to colonise the mouth. The relatively low homology between EfaA and the salivary adhesins, as compared to homology within the group, may explain the loss of the organism's ability to bind components of saliva. However, the fact that the gene has been retained through evolution suggests that it still serves an important function to the cell, possibly mediating coaggregation, or adhesion to some other host substrate(s). Similarly, PsaA of *S. pneumoniae* may promote adhesion to host tissues, particularly in the respiratory tract, but not in the oral cavity. This is more difficult to explain, because of the very high (92.3%) homology to FimA, a known salivary adhesin. The most probable reason is that small changes in the primary amino acid sequence give rise to more significant alterations in tertiary structure, which destroy the active sites necessary for saliva binding. When Oligino and Fives-Taylor (1993) used purified FimA to block binding of *S. parasanguis* to SCHA, they found that only fully renatured protein was effective, indicating that the ability of FimA to bind to its receptor is dependent on a specific three-dimensional conformation of its peptide chain.

4.5.16. Adhesion in the pathogenesis of endocarditis.

It is significant that oral streptococci are a major cause of infective endocarditis, particularly after dental procedures. These organisms must possess virulence factors which allow routine colonisation of the oral cavity, and also opportunistic colonisation of the endocardium. Whether or not the adhesive factor(s) involved are common to both sites is yet to be determined. Animal models have demonstrated that infective endocarditis is far more common following extraction of teeth with periodontal disease than healthy teeth, but that there is no direct correlation between the number of bacteria introduced into the bloodstream during extraction, and the incidence of endocarditis. This suggests that the incidence of infective endocarditis following tooth extraction is affected not so much by the number of bacteria entering the blood stream, as the properties of the organisms, such as their ability to adhere (Moreillon *et al*, 1988; Barco, 1991).

The ability of *E. faecalis* to cause endocarditis is well known, it is the third most common cause of the disease behind oral streptococci and staphylococci, causing 5-15% of cases. In contrast to oral streptococci, the sources of infection are most commonly surgical procedures involving the genito-urinary tract, procedures secondary to childbirth and use of contaminated equipment by intra-venous drug abusers. The ability of *E. faecalis* to cause endocarditis is most likely enhanced by its ability to adhere to heart valves once introduced into the blood stream. Gould *et al* (1975) demonstrated a correlation between an organisms ability to adhere to heart valve tissue in vitro and its ability to cause endocarditis. In this study, enterococci adhered to heart valves more readily than viridans streptococci or staphylococci. Thus enterococci appear to possess an adhesive factor which allows them to attach to the heart valve more avidly than other organisms. It is possible that EfaA might represent such a factor. It has been shown above (section 3.3.) that EfaA is cross reactive with the 37 and 40 kDa antigens of *E. faecalis*, and probably represents the 40 kDa antigen. These antigens have been shown to be endocarditis-specific, ie expressed by

E. faecalis specifically during infection of the heart valve (Shorrock *et al*, 1990). Thus the antigens appear to be somehow involved in endocarditis, and the homology studies on EfaA suggest an adhesive function. It must be pointed out, however, that there is as yet no experimental evidence indicating a role for EfaA. Expression of the 37 and 40 kDa antigens can be induced by growth in serum, suggesting that the antigens are expressed prior to colonisation of the myocardium, which is in agreement with their being necessary to attach to the site of infection. Guzman *et al* (1989) noted that *E. faecalis* strains isolated from urinary tract infections (UTIs) adhered to urinary tract epithelial cells more efficiently than to heart cells, and that strains isolated from endocarditis adhered to heart cells more efficiently than UTI isolates. This suggested that specific adhesive factors were being expressed according to the growth environment, which best allowed cells to colonise the surrounding tissue. Growth in serum enhanced *E. faecalis* adherence to heart cells by up to 8-fold, whilst reducing their association with polymorphonuclear leukocytes (PMNs) by up to 5-fold, suggesting that persistence in the circulation might prime cells for colonisation of heart tissue. It was later proposed that the serum-enhanced adhesion involved carbohydrate-containing moieties on the bacterial cell surface (Guzman *et al*, 1991). Expression of adhesins mediating aggregation of *E. faecalis* cells in response to pheromone have also been shown to be regulated by serum (Kreft *et al*, 1992), moreover, these adhesins mediate binding to eukaryotic cells via RGDS motifs (Arg-Gly-Asp-Ser, Galli *et al*, 1990) which bind to integrins. Thus, persistence in serum stimulates increased expression of a number of virulence factors which could enable *E. faecalis* to colonise the heart valve. No RGD motifs exist within the amino acid sequence of EfaA, nor do any other areas of homology with aggregation substances exist, but since it is reported that some proteins which bind to integrins do not possess RGD motifs (Falkow, 1991; Isberg 1991), EfaA binding to eukaryotic cells should not be eliminated.

4.5.17. EfaA in the pathogenesis of endocarditis.

If EfaA is an adhesin, then it must have a specific substrate. It is unlikely that the substrate is any component of saliva, since enterococci found in the mouth are rarely, if ever, found in the salivary pellicle, but more commonly in the gingival crevice (Gibbons and van Houte, 1975). The similarity between *efaA* and the oral adhesin operons suggests a common evolutionary source for the loci, but clearly both *efaA* and *psaA* have diverged sufficiently to alter antigenic cross-reactivity and substrate specificity. Indeed, the presence of *efaA* may simply be a relic of the common evolutionary source of the enterococci and streptococci, and as such may be vestigial, having lost its ability to bind substrate. Furthermore, the oral streptococci appear to express their adhesins alongside other proteins transcribed from the same operon, and analysis of the genetic material upstream of *efaA* has indicated substantial differences in this region. However, the presence of the 37 and 40 kDa antigens in *E. faecalis* endocarditis has been demonstrated by the use of the purified antigens in serodiagnosis. The antigens appear to remain unexpressed until induction by some component(s) of serum. The expression of a non-functioning protein by an organism would be a wasteful process, and one which would most likely be deleted through natural selection. A more likely explanation is that in *E. faecalis*, the *efaA* operon has become specialised towards an endocarditis-specific function, possibly, but not necessarily, as an adhesin.

4.5.18. Extracellular matrix-binding proteins.

There are many possible substrates for a potential endocarditis adhesin. One of the major candidates being fibronectin. The importance of fibronectin in infective endocarditis is well recognised (Hamill, 1987). Some of its major features in this respect include its ability to bind various eukaryotic tissues such as fibrin, platelets and collagen, as well as prokaryotic cells, including staphylococci and streptococci (reviewed by Westerlund and Korhonen, 1993) and also *E. faecalis* (Shorrock and

Lambert, 1989). Fibronectin is a large fibril-forming glycoprotein, which can exist in either a soluble or insoluble form. In the extracellular matrix, it is present in its insoluble form, promoting cell adhesion to the matrix. Soluble plasma fibronectin circulates in body fluids, binding wherever damage to the endothelium reveals collagen and other extracellular matrix components. In this situation it binds circulating platelets and fibrin, promoting formation of a clot. When such damage occurs on the heart valve, an NBTE forms, consisting of platelets, fibrin and fibronectin. Many bacterial species have been shown to bind to fibronectin, by way of specific surface adhesins such as the FnBPA and FnBPB proteins of *S. aureus* (Signas *et al*, 1989; Jonsson *et al*, 1991) and the environmentally-regulated protein F of *S. pyogenes* (Sela *et al*, 1993; VanHeyningen *et al*, 1993) among others (Westerlund and Korhonen, 1993). These adhesins share common features, including a region composed of three to five repeats of approximately 37 amino acids in length, containing short conserved sequences enriched in acidic amino acids, and two adjacent glycines. Carboxyl side chains of glutamic and aspartic residues in the acidic region have been shown to be essential for binding to fibronectin (McGavin *et al*, 1991). The repeat region of protein F of *S. pyogenes* has been shown to bind to the 30 kDa N-terminus of fibronectin, and it is thought that a second binding domain, close to the repeat section, binds to the C-terminus of fibronectin (Sela *et al*, 1993). Thus many bacteria are capable of binding to fibronectin at the site of NBTE, as the first step in colonisation of the myocardium. The importance of fibronectin binding proteins in pathogenesis has been demonstrated by Lowrance *et al* (1990) who demonstrated that non-fibronectin binding mutants of *S. sanguis* were less virulent in an endocarditis model than the fibronectin-binding wild type. Also, Hanski and Caparon, (1992), showed that mutants of *S. pyogenes* deficient in production of protein F exhibited a 100-fold reduction in ability to colonise cultured respiratory epithelial cells.

It is important to establish that whilst such adhesive processes are important in their own right, the establishment of infection is likely to be multifactorial, probably

requiring many ligand-receptor intractions. Cheung *et al*, (1994) identified a *S. aureus* regulatory locus, *sar*, which is involved expression of a number of cell wall proteins important in the pathogenesis of endocarditis. A *sar*$^-$ mutant strain, deficient in several cell wall proteins, was less adherent to cultured human endothelium than the parent strain, but was still able to bind to sterile vegetations to some extent, despite a reduction in fibronectin binding (Cheung *et al*, 1992; Cheung *et al* 1994).

As mentioned previously, *E. faecalis* has been shown to bind fibronectin (Shorrock and Lambert, 1989), through a receptor which is yet to be fully characterised, and it has also been shown that the ability of bacteria to bind fibronectin is directly related to their ability to cause endocarditis (Lowrance *et al*, 1990). However, the amount of fibronectin bound by *E. faecalis* was approximately 1000 fold lower than that of other streptococci, and the fibronectin binding ability of *E. faecalis* was significantly increased following the introduction of a protein F gene (Hanski *et al*, 1992). Thus it would appear that fibronectin binding is of relatively minor importance in *E. faecalis*. Antiserum raised against the 37 and 40 kDa antigens had no effect upon the binding of *E. faecalis* to fibronectin. This argues against EfaA being involved in fibronectin binding, although the experimental conditions were such that sub-maximal expression of the antigens would be occurring. EfaA does not share any structural similarities with the previously characterised fibronectin binding proteins, which are expressed constitutively. This still leaves the possibility that EfaA, which appears to be expressed only during endocarditis, may represent a specialised adhesin which is induced by some host factor.

Sommer *et al* (1992) have characterised just such an inducible adhesin in *S. gordonii*, which binds to laminin, a protein secreted by endothelial cells as part of the basal laminae. It is not normally exposed, except during damage to the endothelium. A doublet of proteins (142 and 145 kDa) recognised by sera from patients with *S. gordonii* endocarditis, but not by sera from patients with non infective valvulopathies, was found to be lacking in non-laminin binding variants. Expression

of the proteins could be restored by growth in BHI or serum, but was eliminated by subculture in a chemically-defined medium (CDM). Moreover, expression was induced by addition of laminin or collagen I to the culture medium. Since laminin and collagen do not share structural features, the authors suggested that a non-specific event, such as bacterial agglutination, was the signal involved in up regulation. Laminin binding is common amongst streptococci, and may represent one major difference between non-invasive viridans streptococci and their endocarditis-causing counterparts. Switalski *et al* (1987), examined the laminin-binding ability of various streptococcal strains, and found that most isolates from the blood or heart valves of patients with endocarditis expressed laminin receptors, as compared to only a few oral isolates. Furthermore, the tested strains expressed laminin-binding components to a higher extent when grown in BHI as compared to a CDM. These data support a role for laminin binding in the pathogenesis of endocarditis, and the system described by Sommer *et al* (1992), shows obvious similarities to the 37 and 40 kDa antigens of *E. faecalis*, although the proteins are much heavier than those of *E. faecalis*, and it remains to be seen whether EfaA expression can be induced by laminin or collagen. However, in the study by Switalski *et al* (1987) *E. faecalis* exhibited a very low laminin binding ability, thus laminin binding may be of lower significance in *E. faecalis* endocarditis.

Streptococcus pyogenes also expresses a surface protein with affinity for the basal laminae of human cardiac muscle (Winters *et al*, 1993). The 9 kDa glycosaminoglycan-binding protein (GAG-BP) is immunologically cross reactive with similarly sized proteins from other streptococcal strains. These proteins appear to be released by the cells at a localised site of infection. It is hypothesised that they are carried to target organs, such as the heart and kidney, where they accumulate by direct binding. Once located, these proteins elicit an immune response which leads to acute inflammation and tissue injury, as a result of which extracellular matrix components become exposed (Winters *et al*, 1993).

Collagen, another major component of the extracellular matrix, is also exposed at sites of endothelial damage, and so may represent a potential target for adhesion of circulating bacteria prior to endocarditis. Bacteria appear to bind to collagen through both specific adhesion, and indirectly via fibronectin. Fibronectin binds collagen at a site distinct from the bacterial binding site, and so can bind both at once, effectively cross-linking the bacteria to collagen (Hamill, 1987). Patti *et al* (1992) cloned a *S. aureus* specific type II collagen adhesin protein, which may represent an additional direct method of binding to damaged host epithelium, although the authors suggest that the collagen binding activity would be of greater significance in bone infections, where the target tissue is more collagen rich.

4.5.19. Lipoprotein dependent transport systems of Gram-positive bacteria.

In Gram-positive organisms, lipoproteins have been implicated in various recognition and transport systems, some of which show similarity to ATP binding cassette-type (ABC-type) periplasmic binding systems of Gram-negative species (Kolenbrander and London, 1993). Such systems commonly include one or two transmembrane proteins, one or two ATP-binding proteins located on the cytoplasmic side of the membrane and a single high-affinity periplasmic-substrate-binding protein (Tam and Saier, 1993). In the absence of outer membranes, the substrate binding proteins of Gram-positive systems appear to be anchored at the cell surface by means of an N-terminal lipid anchor (Alloing *et al* 1990; Sutcliffe *et al* 1993). Jenkinson (1992) proposed that as well as being involved in uptake processes, lipoproteins in streptococci may be important in adherence-related functions. Specifically, *S. gordonii* mutants deficient in production of a 76 kDa surface lipoprotein were impaired in their ability to aggregate in saliva or serum, and to coaggregate with certain *Actinomyces* species (Jenkinson and Easingwood, 1990; Jenkinson 1992). The EfaA homologues FimA, SsaB and ScaA, which are known to be lipoproteins, have been implicated in adhesion functions. Furthermore, the *scaA* operon has shown similarities to the ABC-type transport systems mentioned above (Andersen *et al*, 1994). It may then be

speculated that the processes of adhesion and transport in Gram-positive species are linked. In the absence of an outer membrane, a Gram-positive organism's substrate-binding protein may encounter its substrate eg saccharides or peptides, in complexed forms, such as on the surface of another bacterium, or in the salivary pellicle, thus acting as an adhesin (Jenkinson, 1994). Whether the lipoproteins have a dual role in both transport and adhesion, or whether the adhesive functions have evolved from transport systems is not known.

These facts further support a role for EfaA in adhesion, and may suggest an extra function related to solute transport. Nucleotide sequence data for regions flanking the *efaA* gene are limited, therefore it is not possible to determine whether a full set of proteins homologous to ATP-binding cassette systems are present adjacent to *efaA*. However, the limited sequence upstream of *efaA* suggests that significant similarities exist (See section 4.2.4.). Even if the ATP-binding proteins are not present adjacent to *efaA*, the potential role for EfaA in adhesion remains.

4.5.20. Avoidance of polymorphonuclear leukocytes.

Guzman *et al* (1989) demonstrated that *E. faecalis* UTI isolates associate with human polymorphonucleocytes (PMNs) more efficiently than endocarditis isolates. Furthermore, growth in serum decreased the association of all *E. faecalis* isolates with PMNs. Clearly, growth in serum affects surface properties of *E. faecalis* cells, decreasing their association with PMNs, whilst at the same time increasing adherence to heart cells. Growth in serum was associated with a decrease in hydrophobicity (Guzman *et al*, 1991a), which could account for some loss of association with PMNs, but since association with heart cells was shown to be enhanced in spite of the decreased hydrophobicity, it was proposed that specific adhesins were present which had a high affinity for heart cells.

It remains a viable possibility that the properties of *E. faecalis* described by Guzman *et al* (1989; 1991a; 1991b) are due to serum dependent expression of the 37 and 40 kDa antigens, and hence EfaA. Increased adhesion to heart cells, decreased association with PMNs and increased hydrophilicity were observed after growth in serum. EfaA has been predicted to be hydrophilic, which may contribute to increased cell hydrophilicity following growth in serum. Guzman *et al* (1991a; 1991b) found that the properties associated with growth in serum were mediated by certain D-galactose- and L-fucose-containing residues present on the bacterial cell surface. Aitchison *et al* (1986) demonstrated that the 37 and 40 kDa antigens of *E. faecalis* were glycosylated, with several residues including glucosyl, mannosyl and particularly fucosyl residues, detected by ligand blotting. This is consistent with the hypothesis that EfaA is the structure displaying these residues.

In summary, sequence analysis of pGP19 has indicated that the immunoreactive expression product described in section 3.5. is encoded by a 924 bp open reading frame, which translates into a 308 amino acid protein, designated EfaA, which has a predicted molecular weight of 34,768 Da. Comparison of the amino acid sequence with the GenBank and EMBL databases revealed similarity with a group of streptococcal proteins, some of which have been shown to be lipoprotein adhesins. On the basis of this homology, the potential role of EfaA in the pathogenesis of endocarditis has been discussed. In the absence of experimental data, no firm conclusions can be drawn as to the function of EfaA, however, it is hoped that the above discussion will provide the basis for further experimental work to this end.

Chapter 5: Regulation of expression of EfaA.

5.1. Previous work.

Shorrock *et al* (1990) showed that expression of the 37 and 40 kDa antigens of *E. faecalis* was under positive control by components of serum, and that expression of the antigens was specific to *E. faecalis* endocarditis. Expression of the antigens, analysed by western blotting, was minimal during growth of the cells in chemically-defined medium (CDM), but could be stimulated by supplementation of the growth medium with small amounts of serum (Aitchison *et al* 1986; Lambert *et al* 1990) with maximal expression during growth in serum. Furthermore, in vivo expression was demonstrated by growth of cells on silastic discs implanted into rabbit peritoneal chambers (Lambert *et al* 1990). To demonstrate conclusively whether expression of EfaA was influenced by serum, a series of northern analyses was performed. Such experiments give an indication of the size and relative abundance of RNA species within the cell population.

5.2. Northern analysis: size of message.

Total cellular RNA was prepared as described in section 2.21.1. Total RNA from cells grown under various conditions were electrophoresed in a 1% denaturing (formamide/formaldehyde) agarose gel, transferred to nitrocellulose membranes and probed with a ^{32}P-labelled internal *efaA* probe. The size of the message to which the probe hybridised was determined by comparison with RNA size markers.

The radiolabelled *efaA* probe hybridised to a mRNA species of approximately 3 kb in length. The stained northern blot and developed autoradiograph are shown in

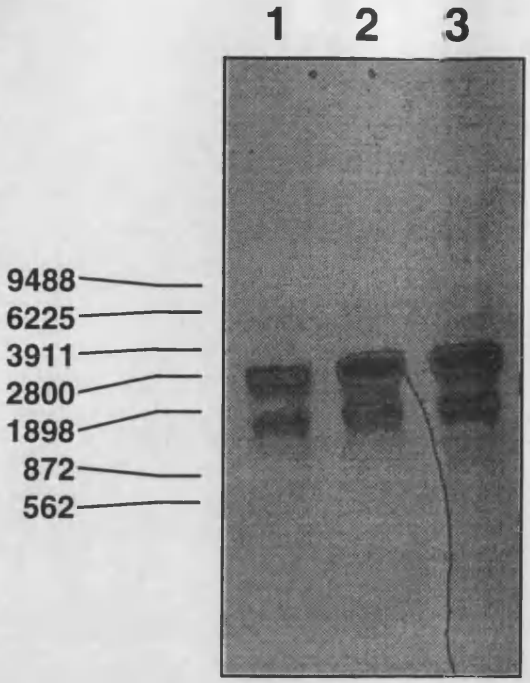

Figure 5.1. Northern blot of *E. faecalis* EBH1 RNA after growth in 1% YE (lane 1), 1% YE/0.1% FCS (lane 2) and 1% YE/1% FCS (lane 3). The blot was stained with methylene blue.

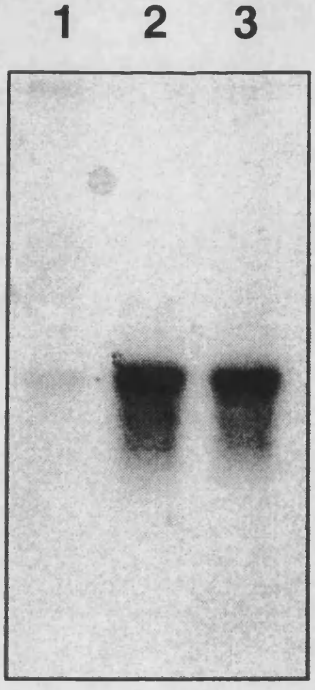

Figure 5.2. Developed autoradiograph of northern blot shown in figure 5.1, probed with a 0.4 kb *Hind*III fragment from *E. faecalis* EBH1 *efaA*.

figures 5.3. and 5.4. Some smearing of the transcript band was evident. This was most likely due to partial degradation of the RNA during extraction and handling.

5.3. Induction of transcription of *efaA*.

To determine whether addition of serum to the growth medium was sufficient to stimulate transcription of *efaA*, *E. faecalis* EBH1 cells were grown in 1% yeast extract overnight, then subcultured 1:100 into three flasks of pre-warmed 1% yeast extract (YE), two of which contained foetal calf serum (FCS) at 0.1% and 1%. Cells were then grown for a further 3 hours, harvested, and RNA was extracted. Equal quantities (5-8 μg) of RNA thus extracted were analysed by northern blotting and hybridisation with the *efaA* probe. The developed autoradiograph is shown in figure 5.2. Band densities, determined by scanning densitometry volume analysis (as described in section 2.25., are shown in table 5.1. To account for any error introduced by uneven loading of RNA onto the gel, densities were normalised by dividing the autoradiograph density by the density of the mehylene blue stained blot.

Table 5.1. Band densities from northern blot autoradiograph (figures 5.1. and 5.2.).

Growth Medium.	Autorad Density	Blot density	Adjusted
1% YE	0.45%	3.62%	0.12
1% YE + 0.1% FCS	27.76%	5.94%	4.67
1% YE + 1% FCS	20.50%	5.23%	3.92

The intensity of the radioactive signal is proportional to the amount of radiolabelled probe which hybridised to the immobilised RNA. Thus the densities of the bands on the autoradiograph are approximately proportional to the quantity of RNA on the northern blot. Thus measurements made in this way are a good estimate

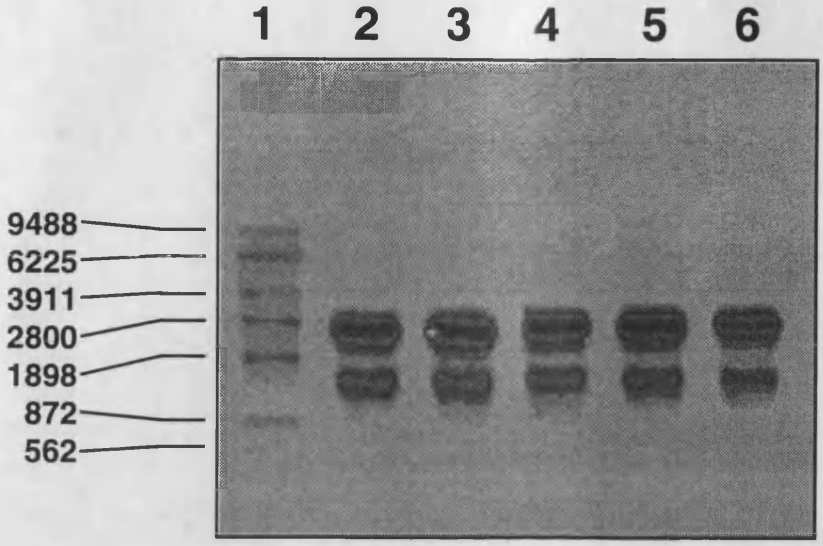

Figure 5.3. Northern blot of *E. faecalis* EBH1 RNA following induction with FCS. Lane 1, molecular weight markers; Lane 2, pre-induction; Lane 3, 15 minutes induction; Lane 4, 30 minutes; Lane 5, 60 minutes; Lane 6, 120 minutes induction.

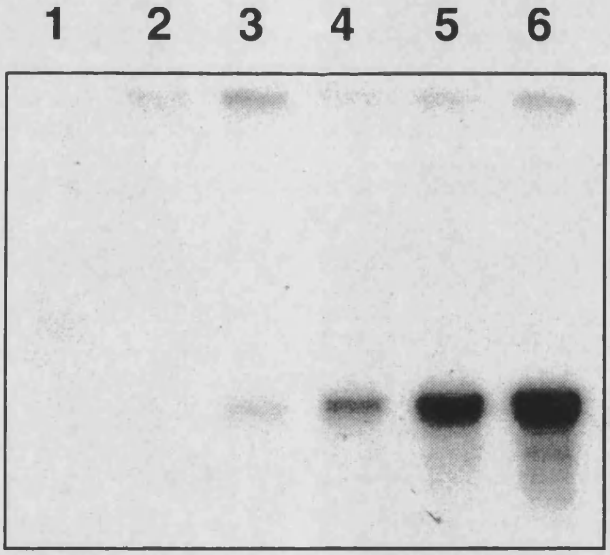

Figure 5.4. Developed autoradiograph of northern blot shown in figure 5.3, probed with a 0.4 kb *Hind*III fragment from *E. faecalis* EBH1 *efaA*.

of RNA quantity, but are not 100% accurate, as will be discussed later (Britten and Davidson, 1985; Anderson and Young, 1985).

The results shown here indicate that transcription of the *efaA* message is minimal during growth in 1% YE, but addition of as little as 0.1% FCS is sufficient to enhance transcription by approximately 35-fold.

5.4. Time course of *efaA* induction by serum.

As a further confirmation of the ability of FCS to stimulate transcription of the *efaA* message, and to characterise partially the response to FCS, the experiment described above was modified. Following overnight growth in 1% YE, cells were subcultured, grown to early log phase (OD_{600} = 0.1), and FCS was added to 1%. Twenty millilitre samples were extracted at time = 0, 15, 30, 60 and 120 minutes, and total RNA extracted as described in section 2.21.1. The stained northern blot and corresponding autoradiograph are shown in figures 5.3. and 5.4. The results were analysed and normalised by scanning densitometry as described in section 5.3, and are shown in table 5.2.

Table 5.2. Band densities of northern blot and autoradiograph (figures 5.3. and 5.4.).

Time after induction	Autorad Density	Blot density	Adjusted
0	0.00	8.02	0.00
15	0.45	8.41	0.05
30	3.64	7.92	0.46
60	16.00	8.06	1.99
120	22.40	6.49	3.45

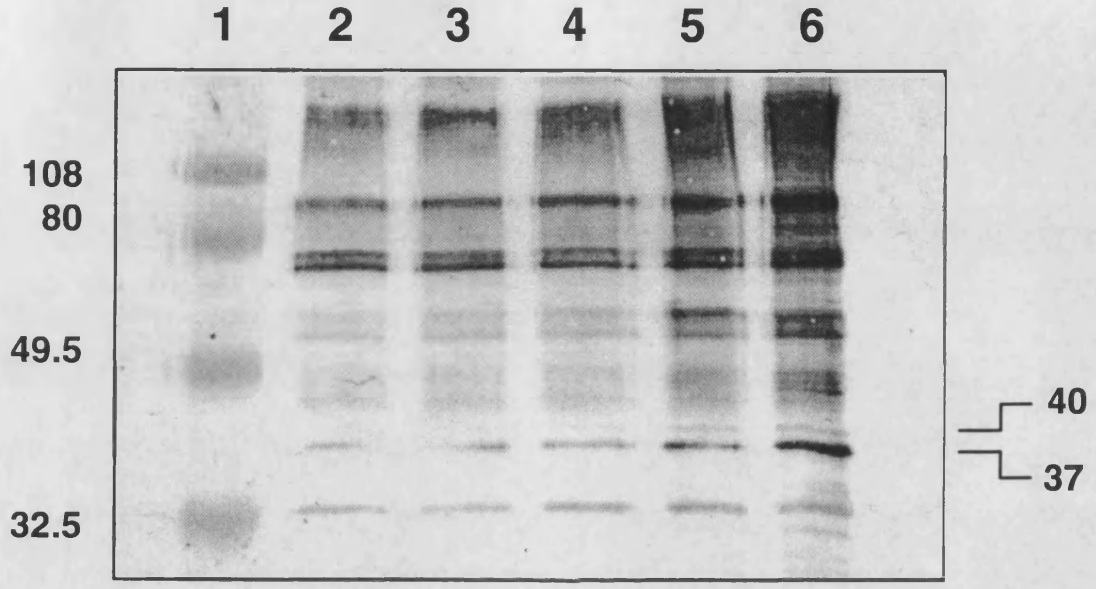

Figure 5.5. Immunoblot analysis of *E. faecalis* EBH1 whole cells following induction with FCS. Lane 1, molecular weight markers; Lane 2, pre-induction; Lane 3, 15 minutes; Lane 4, 30 minutes; Lane 5, 60 minutes; Lane 6, 120 minutes induction.

5.5. Time course of EfaA expression following induction with serum.

The experiments above confirmed that transcription of *efaA* was enhanced following addition of serum to the growth medium. To correlate this finding with expression of protein, cells from the experiment described in section 5.4. were subjected to SDS-PAGE and immunoblotting. Immunoblots were probed with serum from an endocarditis patient with a high IgG titre against *E. faecalis* (GP serum). The immunoblot is shown in figure 5.5. The immunoblot demonstrates that prior to induction, the 37 and 40 kDa antigens are barely visible, but that expression is stimulated following addition of serum. Thus it would appear that the antigens are indeed expressed in response to serum, with the 37 kDa protein expressed first, and the 40 kDa protein appearing slightly later.

5.6. Investigation of the inducing factor(s) in serum.

In order to determine which components of serum were affecting transcription of *efaA*, the serum was pre-treated in a number of ways prior to addition to the culture medium. Pre-treatments were; i) Saturation of the serum with 1mM $FeCl_3$, ii) dialysis against sterile distilled water, to remove low molecular weight species and iii) heating to 65°C for 15 minutes. To investigate the effect any components secreted into the culture supernatant may have had, one batch of cells was harvested and washed in 0.9% NaCl, then the supernatant was replaced with fresh pre-warmed 1% YE prior to addition of serum. Finally, one batch of cells was subcultured into Difco brain heart infusion (BHI), to see whether transcription could be induced by this medium, as suggested by Shorrock (1990).

Cells were grown for 1 hour after induction, then northern analysis and subsequent densitometry of the developed autoradiograph was performed as described

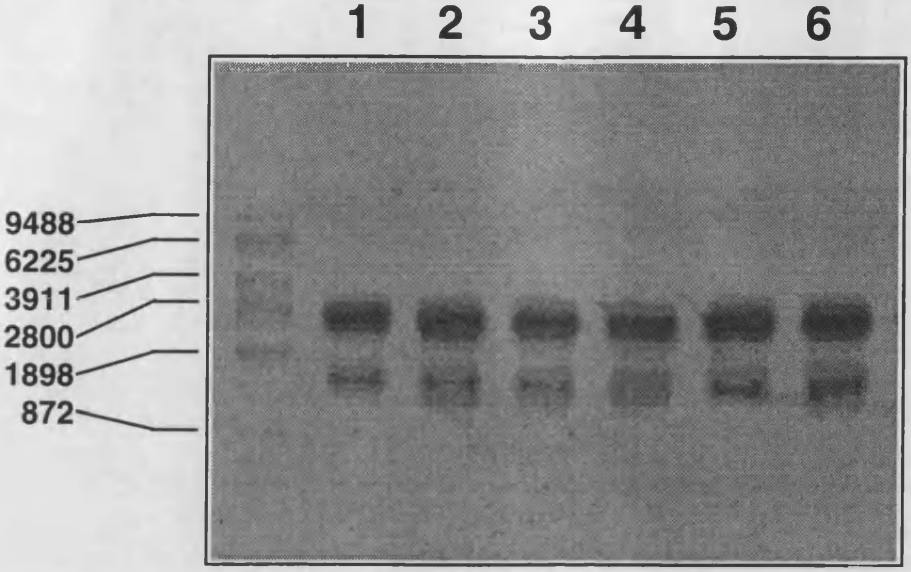

Figure 5.6. Northern blot of *E. faecalis* RNA grown in: Lane 1, 1% YE; lane 2, 1% YE/1% FCS; lane 3, 1% YE/1% FCS (dialysed); lane 4, 1% YE/1% FCS + 1mM $FeCl_3$; lane 5, 1% YE/1% FCS (heated); lane 6, Difco BHI.

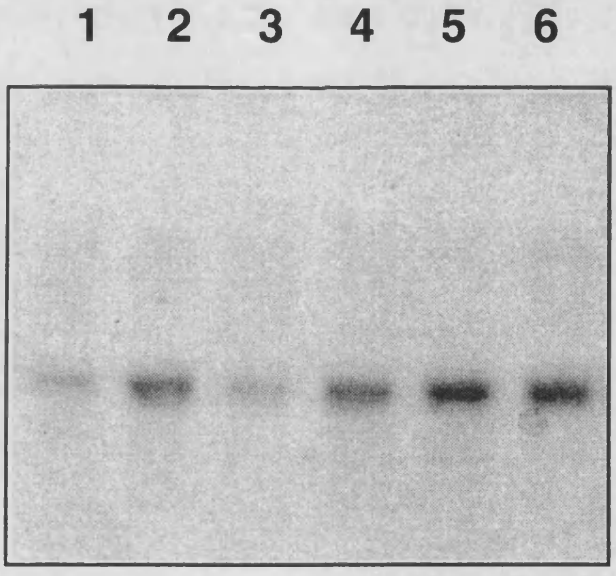

Figure 5.7. Developed autoradiograph of northern blot shown in figure 5.6, probed with a 0.4 kb *Hind*III fragment from *E. faecalis* EBH1 *efaA*.

above. The stained blot and developed autoradiograph are shown in figures 5.6. and 5.7., respectively.

Table 5.3. Band densities of northern blot and autoradiograph in figures 5.6. and 5.7.

Cell treatment.	Autorad density	Blot density	Adjusted
None	0.6%	4.42%	0.14
1% FCS	2.42%	4.60%	0.53
1% FCS (Dialysed)	0.73%	4.54%	0.16
1% FCS + 1mM $FeCl_3$	2.24%	4.41%	0.51
1% FCS (Heated)	3.27%	5.41%	0.60
BHI	3.36%	5.78%	0.58

5.7. Discussion.

5.7.1. Transcript size.

The radiolabelled *efaA* probe hybridised to a 3 kb RNA species. This suggests that *efaA* (927 bp) may be transcribed as part of a polycistronic message. Analysis of DNA regions flanking the *fimA, ssaB, scaA* and *psaA* genes has shown them to be homologous, and it would appear that in all four homologues, other smaller genes are transcribed on the same message as the putative adhesin genes, as discussed in section 4.2.4. Transcript sizes for the *efaA* homologues *fimA* and *psaA* have been shown to be approximately 3.2 kb and 3.0 kb, respectively (Fives-Taylor *et al*, 1991; Sampson *et al* 1994), i.e., very similar to that of *efaA*. These data lend further support to the idea that the five homologous operons share a common evolutionary source, and that each species has diverged, such that the specific functions of the genes involved have become altered.

5.7.2. Induction by element(s) of serum.

The addition of as little as 0.1% of serum to the culture medium was shown to induce transcription of *efaA*. This is in agreement with work by Shorrock *et al,* (1990) who showed that expression of the 37 and 40 kDa antigens was strongly influenced by growth in serum. The amount of *efaA* transcription in the absence of serum was very low, and was sometimes undetectable. Thus it would appear that EfaA is expressed specifically in response to some element of serum. It has been proposed that persistence of *E. faecalis* in the circulation during bacteraemia is necessary for the expression of surface components which allow colonisation and invasion of endothelial cells (Guzman *et al,* 1991b). Since in vitro bacterial growth in serum mimics in vivo persistence in blood, it would appear that EfaA could be one such factor. Furthermore, the similarities which can be drawn between EfaA and the as yet uncharacterised surface structures described by Guzman *et al,* (1989, 1991a, 1991b) suggest that EfaA may be responsible for the serum-dependent changes of *E. faecalis*, as discussed in section 4.5.20.

5.7.3. Time course of transcription induction.

Results shown in table 5.2. show that induction by serum was detectable after 15 minutes, and continued to increase over two hours. The response appears to level off towards 120 minutes. This phenomenon may indicate that a maximal level of transcription is being reached. Alternatively, it may be an artefact of the filter hybridisation method used. A curve relating signal intensity to quantity of RNA is only linear for limited amounts of filter-bound RNA, since at higher levels, the filter-bound sequences may be in excess of the probe. Furthermore, overloading of the filter, such that sequences are not fully accessible to the probe, and overexposure of the autoradiograph, can contribute to inaccuracy (Britten and Davidson, 1985; Anderson and Young, 1985).

5.7.4. Time course of protein expression.

Western blot analysis of *E. faecalis* whole cells following induction with serum, showed an increase in expression of both 37 and 40 kDa antigens, the 37 kDa antigen appearing earlier and more prominently than the 40 kDa antigen during the two hour induction phase. Thus the expression of protein was correlated to enhanced transcription of *efaA* message. It was suggested earlier (section 3.7.1.) that the 37 and 40 kDa antigens may represent unmodified and mature EfaA, respectively. If this is indeed the case, then the following speculative theory may explain the sequential appearance of the two antigens. Initially, following induction with serum, the rate of *efaA* transcription was low, allowing translation of message into EfaA, and rapid conversion to the mature, surface exposed lipoprotein, as seen by the early appearance of the 37 kDa antigen. As induction continued, the amount of cellular *efaA* message increased, so the rate of production of EfaA would also increase, such that processing of EfaA becomes rate-limiting, causing a build up of the 40 kDa antigen. N-terminal sequencing of the 37 and 40 kDa proteins would establish the accuracy of this theory, however, attempts at sequencing were unsuccessful due to contamination by other cell proteins.

The time scale of induction was similar to that seen by Guzman *et al*, (1989), who detected increased adhesion to heart cells, and decreased association with PMNs within 1 hour of serum induction. EfaA protein was detectable in *E. faecalis* whole cells approximately 1 hour after induction, suggesting that it may have been at least partially responsible for these serum inducible changes in *E. faecalis* properties.

5.7.5. The nature of the inducing factor.

The results shown in table 5.3. indicate that the inducing factor was present in both serum and BHI, was resistant to heat (BHI was sterilised by autoclaving) and was apparently not related to iron levels. The ability of serum to induce expression was removed after overnight dialysis against sterile double distilled water, indicating that

its molecular weight was smaller than 10-12 kDa. Changing the culture supernatant prior to induction had no effect, thus induction was probably not due to the action of extracellular factors acting on serum. Thus the nature of the inducing factor is still unknown, other than that it is heat stable, and no larger than 12 kDa. If the inducer is a peptide, then its heat stability suggests either that it does not have appreciable tertiary structure, since this would be lost during autoclaving, or that the region of the molecule responsible for induction is not involved in the tertiary structure of the molecule.

5.7.6. Gene regulation in response to the environment: Virulence factors.

Pathogenic bacteria have a highly adapted lifestyle which requires survival and multiplication within a living organism. The particular strategies used in this respect distinguish pathogens from innocuous flora or bacteria which can infect only compromised hosts. The ability to proliferate in various niches within the host, and to survive the transition to and from the host, requires considerable adaptability on the part of the pathogen, and such pressures have probably directed the evolution of specialised systems regulating the expression of virulence factors. A virulence factor need not be designed to damage specifically the host, but more often allows the pathogen to compete with the host for nutrients, or to survive within the hostile environment of the host's tissues. A successful bacterial pathogen must maintain a balance in its pathogenic cycle which optimises the survival and multiplication within the individual host as well as within the population as a whole. Disease is merely a manifestation of the complex interaction between host and pathogen (Mekalanos, 1992), the degree of host injury does not always correlate with success of the pathogen, and death of the host is usually considered counterproductive to the pathogen. Indeed, for virtually all pathogens, subclinical infections outnumber those which cause overt disease (Dirita and Mekalanos, 1989). The fact that most bacterial pathogens do not constitutively express their virulence factors demonstrates the

important role that tight regulation of these factors must play in the success of a pathogen (Dirita and Mekalanos, 1989).

Often virulence factors are under the control of a global regulatory system, such as the *sar* locus of *S. aureus* (Cheung *et al*, 1992; 1994). These systems are defined by a common environment sensing system, which influences expression of a wide range of virulence determinants. Thus bacterial pathogenesis is a process whereby the pathogen is constantly sensing its environment and responding appropriately (Miller *et al,* 1989; Finlay and Falkow, 1989). There are many potential cues for initiation of virulence gene expression, including specific host factors such as temperature and pH (Pepe *et al*, 1994) and iron concentration (Griffiths, 1991).

5.7.7. Gene regulation in response to the environment: Signal transduction.

A two-component model for the process of environmental sensing and signal transduction in bacteria has been proposed (reviewed by Parkinson, 1993). The model consists of a cytoplasmic membrane-spanning sensor/transmitter protein and a cytoplasmic receiver/regulator protein. Upon sensing the appropriate environmental signal, the transmitter protein activates its corresponding cytoplasmic receiver, which in turn can exert a regulatory effect upon cell processes. Receiver proteins usually contain DNA binding regions, so it is likely that activation alters the ability of this region to bind to the operator region of the relevant gene(s).

Such two-component mechanisms lend flexibility to the system whereby a single sensor protein can interact with multiple regulatory components, allowing large scale changes in cell function in response to a single environmental cue (Kofoid and Parkinson, 1988; Ninfa *et al,* 1988). Conversely, specificity is of great importance, since an organism may have approximately 50 transmitter-receiver pairs, and inappropriate cross talk between them is minimal (Parkinson 1993).

Alterations of gene expression at the transcriptional level are usually achieved by binding of regulator proteins to areas of DNA known as operator sites, which either stimulate or inhibit RNA polymerase binding and subsequent transcription of the particular genes. Where the binding of a regulator protein to an operator site enhances transcription, the gene is said to be under positive regulation, and conversely, when a regulator inhibits transcription, the gene is under negative regulation. In either situation, alterations in the conformation of the regulator protein dictated by various cellular processes can either enhance or inhibit transcription of a particular gene (Alberts *et al* 1989).

5.7.8. The response of E. faecalis to serum.

The results in sections 5.3-5.6. indicate that expression of EfaA is stimulated by exposure of the cells to serum, an event which mimics growth in the circulation. The transition of a bacterium from a site of primary infection to the circulation represents a change in environment for the organism. Serum contains numerous host defence mechanisms, and an invading cell must evade these, whilst competing for available nutrients. To ensure survival, an organism must undergo certain changes once introduced into the circulation. Such changes must ensure survival in the host, and as such, may be termed virulence factors. Other serum-induced virulence-related events have been characterised in *E. faecalis*. Kreft *et al* (1992) found that synthesis of aggregation substance was induced by some component(s) of serum. Aggregation substance normally causes clumping of *E. faecalis* cells in response to sex-pheromones, however, Galli *et al* (1990) noted that the amino acid sequence of aggregation substance contained RGD motifs, which are known to bind to receptors on eukaryotic cells. Kreft *et al* (1992) confirmed that aggregation substance could bind to eukaryotic cells, and showed that expression could be induced by serum. Subsequently, Chow *et al*, (1993) examined the virulence of several *E. faecalis* strains harbouring various mutations of pAD1, and showed that expression of aggregation substance, along with hemolysin production was associated with increased mortality in

an endocarditis model. Thus, pAD1-encoded aggregation substance may be a virulence factor. As has been described above, Guzman *et al* (1989, 1991a, 1991b) have shown that the surface properties of *E. faecalis* in relation to its binding to endothelial cells and interaction with PMNs are profoundly affected by growth in serum.

5.7.9. Expression of EfaA in response to serum.

Since EfaA is expressed during growth in serum, then it may be assumed that its expression is required for optimal growth in vivo, and so EfaA may be termed a virulence factor. Its function has yet to be determined, but many of the potential functions discussed above, such as adhesion to endothelial cells, and avoidance of PMNs would give the cell a greater chance of survival in a hostile host environment. It seems likely then, that the ability of *E. faecalis* to sense its surroundings is of importance during the course of infection. Induction of expression was detectable after the addition of only 0.1% FCS, so the sensing mechanisms would appear to be responding to a specific element within serum, possibly some nutrient not found elsewhere in the host. The inducing factor appears to be smaller than 10-12,000 Da, since it was removed from serum by dialysis. Furthermore, the inducer is heat stable, which reduces the likelihood of it being a complex protein. Studies of the *scaA* operon have revealed similarities with periplasmic binding proteins of gram negative organisms, suggesting that this particular homologue of EfaA may be involved in some form of membrane transport in *S. gordonii* (Andersen *et al*, 1994), as well as mediating coaggregation with *A. naeslundii*. If EfaA also performs a transport function, then there may be some mechanism by which the presence of substrate switches on the uptake function. It may be then that the inducing factor in serum is a nutrient not available in other growth media, such as oligopeptides, or sugars, both of which are transported by lipoproteins in other species (Pearce *et al*, 1992; Sutcliffe *et al*, 1993). The fact that DNA flanking the *efaA* gene differs in certain elements present upstream of *scaA* may indicate a loss of the ability of EfaA to participate in transport,

but not a loss in the ability to respond to the inducing factor. Such a situation would allow EfaA to evolve to some other function specific to presence in serum, such as adhesion to certain target molecules. The cloning and sequencing of more DNA upstream and downstream of *efaA* will be required in order to determine whether genes homologous to ATP-binding cassette operons are present, as is the case for *scaA* of *S. gordonii* (Andersen *et al*, 1994).

Similarities between EfaA and the putative laminin binding protein of *S. gordonii* (Sommer *et al*, 1992), are apparent. The proteins were recognised by sera from patients with endocarditis, but only weakly by sera from patients with non-infected valvulopathies, and expression was enhanced by growth in serum, BHI, laminin or collagen. The induction studies performed on *efaA* did not include addition of elements of the extracellular matrix, so comparisons cannot be made in this respect. However, since laminin and collagen do not share any common structural features, the authors suggested that the positive signal may be some non-specific event related to laminin or collagen, which could also be the case for induction by serum or BHI.

In summary, *efaA* appears to be transcribed as part of a 3 kb message, a size which is very similar to that determined for homologous genes. Its transcription is strongly influenced by low molecular weight components of serum. These data are in agreement with previous findings, and support the hypothesis that EfaA is expressed when *E. faecalis* makes the transition from a primary infection site to the circulation, possibly priming the organism for colonisation of the endocardium.

Chapter 6: Concluding discussion.

Enterococcus faecalis endocarditis is causing increasing concern, carrying significant mortality, and often leaving permanent damage to the heart valve. Treatment is increasingly difficult, since *E. faecalis* is rapidly becoming resistant to many drugs. The continued widespread use of antibiotics in hospitals means that emergence of resistance is likely to continue until all bactericidal drugs are of little use. In such cases, the prognosis of enterococcal endocarditis is almost certainly death. In the future, treatment may rely on a whole new generation of antimicrobials. In order to design such treatments, a detailed understanding of the pathogenic mechanisms of the causative organisms is required.

Growth in serum, approximating to growth in the circulation, is known to enhance the adhesion of *E. faecalis* to heart cells. Furthermore, an examination of the antigenic profile of serum-grown *E. faecalis* cells reveals enhanced expression of certain surface proteins. These antigens appear to be specific to endocarditis, and so may contribute to pathogenicity of *E. faecalis* in this condition. Characterisation of these serum-regulated surface components and determination of their role in endocarditis may provide new targets for therapy of this disease.

This study was designed to exploit the antigenicity of two serum-regulated proteins, the 37 and 40 kDa antigens, in order to isolate clones containing their structural genes. A gene expressing an approximately 40-kDa protein which was antigenically cross-reactive with the 37 and 40 kDa antigens was cloned, suggesting that they may represent different forms of the same protein. Nucleotide sequence analysis revealed an open reading frame encoding a protein with a predicted molecular weight of 34,768 designated *Enterococcus faecalis* antigen A (EfaA). Confirmation of the relationship of the 37 and 40 kDa antigens to each other and with EfaA requires amino acid sequence analysis of the purified antigens. There is a lipoprotein-type

consensus sequence at the N-terminus, suggesting EfaA is anchored via an aminoacyl group after cleavage of a leader sequence. Incomplete processing of the leader peptide may provide one possible explanation for the occurrence of the two antigens in whole cell extracts.

EfaA shows 55-60% homology to a group of streptococcal adhesins, suggesting a role in adhesion. Recent studies on genes flanking *scaA* in *S. gordonii* indicate a similarity with lipoprotein-dependent solute transport systems. In gram positive species it has been proposed that the solute-binding component of these systems may act as an adhesin when its substrate is encountered on a solid surface. The finding that EfaA expression is regulated at the transcriptional level by components of serum is consistent with it being involved with solute transport systems, since expression of the transport proteins is often enhanced by their substrate. The nature of the inducing component in serum remains to be elucidated, however, it appears to be heat stable, and of low molecular weight. In this respect, a connection with pheromone sensing mechanisms of *E. faecalis* has been suggested (G Dunny, personal communication).

Identification of EfaA represents the discovery of common ground between the enterococci and the oral streptococci, which between them account for the large majority of all cases of infective endocarditis. These data highlight the possibility that the adhesins identified amongst the oral streptococci should be investigated in the pathogenesis of streptococcal endocarditis.

6.1 Further work.

At present, no experimental evidence exists to establish the function of EfaA in infective endocarditis. A major step towards this end would be the creation of an *E. faecalis* EfaA-deficient mutant. It would then be possible to compare the mutant with the parent strain in an experimental endocarditis model. Furthermore, the role of EfaA in adhesion could be investigated by making similar parent/mutant comparisons

in binding assays, using substrates such as intact cardiac endothelium, blood clots, fibronectin, and other extracellular matrix components.

Knowledge of the role of EfaA in the pathogenesis of *E. faecalis* endocarditis may indicate new treatment strategies, perhaps based on anti-adhesion agents or vaccines. This would represent a major advancement in the therapy of *E. faecalis* endocarditis.

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APPENDICES

Appendix A: Amino acids.

Amino acid.	Abbreviation.	Code Letter.	Mass.	Properties (hydrophilicity).
Alanine	Ala	A	89.09	Neutral, (-0.5)
Arginine	Arg	R	174.2	Basic, (3.0)
Asparagine	Asn	N	132.1	Neutral, (0.2)
Aspartic acid	Asp	D	133.1	Acidic, (3.0)
Cysteine	Cys	C	121.12	Neutral, (-1.0)
Glutamic acid	Glu	E	147.13	Acidic, (3.0)
Glutamine	Gln	Q	146.15	Neutral, (0.2)
Glycine	Gly	G	75.07	Neutral, (0.0)
Histidine	His	H	155.16	Basic, (-0.5)
Isoleucine	Ile	I	131.17	Neutral, (-1.8)
Leucine	Leu	L	131.17	Neutral, (-1.8)
Lysine	Lys	K	146.19	Basic, (3.0)
Methionine	Met	M	149.21	Neutral, (-1.3)
Phenylalanine	Phe	F	165.19	Neutral, (-2.5)
Proline	Pro	P	115.13	Neutral, (0.0)
Serine	Ser	S	105.09	Neutral, (0.3)
Threonine	Thr	T	119.12	Neutral, (-0.4)
Tryptophan	Trp	W	204.22	Neutral, (-3.4)
Tyrosine	Tyr	Y	181.19	Neutral, (-2.3)
Valine	Val	V	117.15	Neutral, (-1.5)

Hydrophilicity value according to Hopp and Woods (1981)

Appendix B: Codon Usage in Streptococci.

Codons per thousand base pairs (Wada *et al*, 1992).

ARG:	CGA	3.2	ALA:	GCA	26.1	GLN:	CAA	33.8
	CGC	5.6		GCC	13.2		CAG	10.6
	CGG	1.7		GCG	8.5			
	CGU	15.2		GCU	37.8			
	AGA	7.0						
	AGG	1.6						
LEU:	CUA	9.1	GLY:	GGA	15.1	HIS:	CAC	5.9
	CUC	7.4		GGC	10.9		CAU	9.6
	CUG	6.9		GGG	6.7			
	CUU	18.2		GGU	28.3			
	UUA	20.1						
	UUG	18.9						
SER:	UCA	16.7	VAL:	GUA	13.7	GLU:	GAA	53.9
	UCC	3.5		GUC	12.2		GAG	16.8
	UCG	3.0		GUG	9.7			
	UCU	16.0		GUU	29.4			
	AGC	9.4						
	AGU	13.9						
THR:	ACA	25.7	LYS:	AAA	55	ASP:	GAC	19.4
	ACC	13.1		AAG	27.8		GAU	44.8
	ACG	7.6						
	ACU	23.9						
PRO:	CCA	17.5	ASN:	AAC	20.2	TYR:	UAC	15.0
	CCC	2.2		AAU	39.1		UAU	29.8
	CCG	4.4						
	CCU	12.3						
CYS:	UGC	1.0	PHE:	UUC	12.5	ILE:	AUA	5.6
	UGU	2.3		UUU	23.9		AUC	17.0
							AUU	31.0
MET:	AUG	17.8	TRP:	UGG	9.8	TER:	UAA	1.2
							UAG	0.4
							UGA	0.2

Appendix C: Molecular Weight Markers.

For agarose gel electrophoresis, markers consisted of *Eco*R1/*Hin*d III-cut lambda DNA(Northumbria Biologicals). The digest consists of DNA fragments of the following sizes (Base pairs):

1:	21226	8:	1584
2:	5148	9:	1375
3:	4973	10:	947
4:	4268	11:	831
5:	3530	12:	564
6:	2027	13:	125
7:	1904		

For SDS-PAGE and immunoblotting, pre-stained markers (Sigma) consisted of;

Protein	Molecular Weight
Phosphorylase B	106 kDa
Bovine serum albumin	80 kDa
Ovalbumin	49.5 kDa
Carbonic anhydrase	32.5 kDa
Soybean trypsin inhibitor	27.5 kDa
Lysozyme	18.5 kDa

For denaturing agarose gel electrophoresis, RNA markers (Sigma) consisted of (bp);

1:	9488	5:	1898
2:	6225	6:	872
3:	3911	7:	562
4:	2800	8:	363

Appendix D: Media and Solutions.

Where appropriate, media and solutions were sterilised by autoclaving for 20 minutes at 15 lbs/sq inch (121°C) on a liquid cycle.

BHI: Bacto brain heart infusion agar and broth (Difco.)

Chloroform:isoamyl alcohol: Chloroform mixed with isoamyl alcohol 24:1 (Amresco)

DNA denaturing buffer: 0.2M NaOH, 0.2mM EDTA, pH 8.0.

DNA loading buffer: 0.5% SDS, 25% glycerol, 0.25% bromophenol blue, 0.05M EDTA.

LB medium (Luria-Bertani Medium): (per litre) 10g Bacto tryptone (Difco), 5g Bacto yeast extract (Difco) and 10g NaCl, pH adjusted to 7.0 with NaOH. LB plates were made from 1 litre of LB medium plus 20g agar (Difco).

NZCYM broth: 1 litre of NZY broth plus 1g casamino acids (Difco), pH 7.0.

NZY Broth: (per litre) 5g NaCl, 2g $MgSO_4$, 5g Bacto yeast extract (Difco) and 10g NZ amine (ICN) pH 7.5. NZY plates were made from 1 litre of NZY broth plus 20g agar (Difco).

SB (Super Broth) Per litre: 35g Bacto tryptone (Difco), 20g Bacto yeast extract (Difco), 5g NaCl pH7.5

SSC (1x): 0.15M NaCl, 0.015M trisodium citrate pH 7.0

SM (Phage dilution buffer): (per litre) 5.8g NaCl, 2g $MgSO_4$, 50ml 1M Tris pH 7.5 and 5ml 2% gelatin.

SOC: 2% Bacto tryptone (Difco), 0.5% Bacto yeast extract (Difco), 10mM NaCl, 2.5mM KCl, 10mM $MgCl_2$, 10mM $MgSO_4$, 20mM glucose.

TB (Terrific broth): (per litre) 5g NaCl and 10g Bacto tryptone (Difco), pH adjusted to 7.4 with NaOH.

TBE (1x): (per litre) 10.8g Tris base, 5.5g boric acid, 0.93g $Na_2EDTA.H_2O$, pH 8.3

TBS: 0.9% W/v NaCl in 10mM Tris HCl pH 7.4.

TBS-Tween: 0.3% W/v Tween 20 in TBS.

TE: 10mM Tris-HCl, 1mM EDTA pH 7.0

TE Glucose: 25% W/v glucose, 100mM Tris, 10mM EDTA pH 7.0

Tris-Phenol: Phenol/0.1% hydroxyquinolone, equilibrated with 0.5M, then several changes of 0.1M Tris HCl pH 8.0 until pH is greater than 7.8.

Phenol/chloroform: Tris Phenol : Chloroform : Isoamyl alcohol 25:24:1 (Amresco)